

High time for the circuses to stop

Basic understanding of the Solar System is the poor relation in most space programmes, but the US National Aeronautics and Space Administration should promptly redress the balance by scrapping its planned space station.

THE skirmish two weeks ago between the director of the US National Institutes of Health (NIH) and the administrator of the US National Aeronautics and Space Administration (NASA) is more probably proof of jockeying for position in the present budget round and, more important, that due to begin next January than a sign that the administration is about to change tack on its strategy on space exploration. But Dr Bernadine Healy is correct to complain (see *Nature* 358, 441; 1992) that what NASA calls biology is not what others think it is. Much of what NASA does is ancillary to its practice of sending people into space: how does the human frame function in these unusual circumstances? Just as Antarctic research has been a spur to the understanding of human behaviour in the cold, so NASA's activities will throw light on people's behaviour beyond the atmosphere and normal gravity. But the goal is not so much understanding as the furtherance of NASA's grander goals. And although studies of the behaviour of plants or of electrophoretic systems in near-zero gravity are intrinsically interesting, only dreamers and publicists think them the harbingers of new (and profitable) industrial enterprises in orbit.

Healy joins a host of respectable people, many of them NASA people such as Dr Riccardo Giacconi, the director of the Space Telescope Research Science Institute, in believing — and then openly saying — that NASA is unreasonably preoccupied with the means and not the ends, with sending people aloft rather than with the durable exploration of the Universe, ostensibly its purpose. That view is reflected even in minor management matters: it is not merely a bad joke, but a waste of potentially valuable data, that the General Accounting Office had to complain some months ago that archives of magnetic tape were kept in conditions contributing to their rapid deterioration. More to the point, with every budget squeeze (the reasons for which are widely appreciated) there is a further hunt for ways in which basic science projects can be trimmed back so as to keep the manned spaceflight programme as nearly as possible intact.

NASA is in much the danger of the dog in Aesop's fable who grabbed at the image of a bone in a stream it was crossing, losing both the image and its object, which had been in its mouth. It skimps on basic understanding for the sake of circuses, but it is then tormented (as since the Challenger accident five years ago) by mishaps indicative of heroic attempts to stretch resources farther than they can

be made to go. Last week's satellite whose tether failed to unwind (see *Nature* 358, 526 & 529; 1992) is but the most recent embarrassment. The difficulty is obvious, a version of 'Catch-22'. NASA's claim on public and congressional attention rests on its reputation that daring projects are competently carried through. In the year of Columbus especially, there is budgetary mileage in great projects beyond the Earth; but, by the converse of the same test, disappointment breeds disillusion, and budgets shrink still faster, making the conversion of dreams into reality still more chancy.

Everybody's interests, NASA's included, would be better served if this US budget cycle triggered a reappraisal. Of course, nobody now disputes that flights beyond the atmosphere by people have come to stay. Of necessity, given the long lead-times of these programmes, for several decades hence the venturers will usually be from the United States. So why the hurry? Is it simply that, without a sense of urgency, there would be no budget at all? That is a thoroughly discreditable but also, sadly, a credible explanation of the heady talk of celebrating the beginning of the next millennium with a landing by people on Mars. It would be far preferable that NASA should acknowledge that the space it wishes to see explored by people will be there for a good part of the rest of time, and that the difficulties will shrink as the decades pass.

The planned space station, called 'Freedom', illustrates the point (and NASA's budgetary and strategic difficulties) almost too aptly. Conceptually, it is a project whose grandeur derives from its lack of purpose, as does that of the pyramids of Giza. The best that can be said of it is that it will be big enough to be flexible enough to allow zero-gravity experiments that people have not yet had the wit to devise. When a lot more has been learned of the capabilities and limitations of humans in thick plastic suits, it may also become (with further engineering development) a reassembly point for payloads and rockets aimed at places in the Solar System not yet specified. In principle, it could also be a prolific source of data about the Solar System and beyond — but guess what will be cut when the project, already suffering from the skimpings and delays that budget-cutting brings, is still further attenuated?

Must the circus go on? Especially when the federal deficit is somewhere between \$350 billion and \$500 billion a year? And when the initial unwillingness of overseas partners in the venture (notably in Europe and Japan) will



turn to fury when, as seems likely on present trends, further delays and skimpings will mean overseas development spending has also served no purpose? The best course for NASA would be a decision that circuses are an unseemly means to an admittedly laudable end, that the sooner Freedom is scrapped the better, and that NASA will henceforth cherish the exploration of space in the only sense that matters in the long run, by accumulating the understanding of what the Solar System is like (and how it got like that) on which people's comfort, possibly survival, increasingly depends. And whatever became, for that matter, of the ambitions, current as recently as June this year at Rio de Janeiro, that NASA should play an even fuller part in the understanding of the Earth? The reasons why NASA is unlikely to stumble of its own accord into common sense will be widely appreciated, but a budget-conscious Congress could be a powerful stimulus. □

Slump in seed-corn

British enrolments in science courses are responding to market forces — and falling.

IF the British understood what is happening to them, gloom about the prospects of economic improvement would be more than matched by last week's news that applications for places on science courses in higher education have fallen again this year, although most institutions are ready to take more science students than ever and the schools are generating record numbers of students qualified (in other fields) for higher education. One spokesman says that some universities have as many vacant places as there are students seeking to occupy them. Nobody should be surprised. What the figures show is merely what every economist has been saying since Adam Smith's time — that supply and demand will match each other at a certain market-clearing price. Britain's problem is a low market-clearing price for technical skill established over the past decade.

Young people choosing careers are not influenced only by the salaries in prospect, either at the beginning or averaged over a career. Esteem (and even self-esteem) is important. Why else do able people who might hope to become judges or tycoons keep the world's orchestras playing, or spend months doing nothing in the hope of entertaining others by occasionally reciting Shakespeare at them? The growing army of the world's welfare workers is similarly motivated. It is not unreasonable that science should traditionally have been partly sustained by similar calculations. It is great fun to learn something for the first time ever, and sometimes there are also prizes. But for a decade in Britain, esteem and self-esteem have been so well mixed with indignity and public humiliation that they have ceased to carry weight, while the tangible benefits of a career in science (at least in the universities) have, breath-takingly, been reduced.

Plainly, the market's message has been accurately conveyed. But it may not be the message the market's managers

had intended. Even in the Thatcher era, there was no reason to suppose the government intended the supply of technical people to fall; rather, it took the supply for granted, but believed that a little (or even a lot of) bullying would make it collectively more productive. That was a curious lapse for a government persuaded of the efficacy of the market. The government's successor has been talking differently in the few months since its election. It could do worse than begin at the most obvious place, by paying graduate students better. The rub is that it will take at least as long for the supply to grow again as it has taken for it to shrink. □

Managing Rio's treaties

The Carnegie Commission would like to see better management of international environmental research.

WHATEVER happened to the two treaties on the environment, signed with all that pomp and clamour at Rio de Janeiro less than three months ago? Perhaps attention-spans have snapped. It is also certain that the international organizations with a finger in the environmental pie are hard at work winning as large a slice of it as they can. But it is far from clear how the two treaties, on global warming and biodiversity, will be given the technical stuffing they need. So it is timely that the Carnegie Commission on Science, Technology and Government should have just published a study advocating clearer management of the international research effort in prospect, (*International Environmental Research and Assessment: Proposals for Better Organization and Decision Making*).

So far as it goes, the report makes sense. While acknowledging the special competence of the international specialized agencies (the UN Environmental Programme, the World Meteorological Office and the like), it concludes that all of them are too specialized to serve even as effective coordinators of research. That is surely the case. As a better model, the study commends the international network of agricultural research institutes run by the Consultative Group for International Agricultural Research (CGIAR), chiefly (but not exclusively) supported by the World Bank and the Ford and Rockefeller Foundations. Carnegie even has an acronym ready: CGREEN, in which the last four characters are the first two of "research" and "environment" in series.

The idea is a good one, but some pieces are missing. CGIAR is a good model for running the network of laboratories there will have to be if accurate environmental assessments are to be carried out, but it is a management device and not a mechanism for continuing and often controversial assessment. For that to work, and have political clout, only a small college of independent people recognizable as such will serve. This journal has sung the praises of the UN Scientific Committee on Energetic Atomic Radiation (UNSCEAR) because, over 35 years, it has won an enviable reputation in a not unconnected field. □

African National Congress drafts blueprint for South African science

Cape Town. The African National Congress (ANC) has released its new science and technology policy, a document aimed at reversing what the organization claims is the apartheid era's legacy of directing "the benefits of technology...to the white minority at the expense of the majority." Because the ANC is considered likely to emerge as South Africa's new government, this plan may well direct the nation's science and technology policy over the next decade.

The new policy focuses on education as the essential ingredient to the growth and development of a scientifically literate and technically able society. It promises high-quality programmes in science, mathematics and technical education at secondary and higher levels, including worker training and education, for South Africans of all races. There are almost no qualified science or technical teachers in black schools because of a shortage of qualified instructors.

It is not quite clear, however, how the ANC expects to recruit enough mathematics and science teachers to reach its goal. In 1989 (the last year for which figures are available) the country's 22 universities produced only 92 physics, 171 chemistry and 216 mathematics majors between them.

The policy supports the growth of indig-

enous technologies, especially by encouraging increased spending on research and development by the private sector, but does not provide details on how to achieve this aim. The ANC states that its policy objectives "do not require additional state funds to be directed towards [science and technology] in the short-term, since they can be achieved through a more efficient and equitable use of the resources already allocated". For example, the annual state subsidy made to the Atomic Energy Corporation for uranium enrichment is roughly equal to the combined grant made to the six statutory councils (US\$238 million in 1992). Abolishing this would effectively double the resources available for science and technology development.

The ANC intends to emphasize research fields with higher market potential, such as micro-electronics, information technology, biotechnology and new materials. But it also pledges to "retain a basis of fundamental research which is internationally recognized and relevant to the long-term needs of the country". It also wants to foster cooperation with other southern African countries in science and technology.

Another stipulation of the policy is the creation of affirmative action programmes

to increase the numbers of scientists and engineers of all races, both men and women. In 1988, 82 per cent of scientists and 96 per cent of engineers in South Africa were white, a consequence of an education system that produces one mathematics and science graduate for every 10,000 black school entrants. Roughly two-thirds of the science graduates are male, a proportion that rises to 98 per cent for engineering graduates.

Among its other proposals, the ANC policy recommends an independent Office of Technology Assessment to monitor technological progress and suggests the establishment of either a Ministry of Research and Technology functioning at cabinet level or a powerful interdepartmental committee. The latter option would not present any significant departure from the present system, in which funds are allocated by a cabinet committee advised by the Scientific Advisory Council.

Commenting on the document, Khotso Mokhele, recently appointed vice president responsible for programmes at the Foundation for Research Development, said that he felt encouraged by "the fact that the ANC has made S&T such a high priority within their policy debate".

Michael Cherry

NSF opens up long-range plan to public comments

Washington. Caught off-guard by a directive from Congress to do more for US industry, the National Science Foundation (NSF) has decided to hold a series of public meetings to find out what scientists think. Last week, at a meeting of its National Science Board, NSF announced the creation of a 15-person Commission on the Future of the National Science Foundation that will spend three months on such issues as whether it is "appropriate" for NSF to broaden its mandate beyond basic research to support technology and stronger links with industry.

Although the issue of NSF's role in supporting industry has been on the table for months as NSF prepared its strategic plan, language in the Senate version of the bill that sets the agency's budget for 1993 (passed on 3 August) has forced the agency to confront the issue. The Senate instructed NSF to take a "more activist role" in transferring technology from US universities to industry, listing half-a-dozen ways in which it should do this and requiring NSF to revise its strategic planning accordingly.

Walter Massey, the NSF director, says that he is "very disappointed" with the Sen-

ate appropriations bill — which, besides providing detailed instructions on how NSF should spend its money, would give the agency barely enough to cover the cost of inflation. He said he has requested a meeting with Senator Barbara Mikulski (Democrat, Maryland), the chairwoman of the appropriations committee that oversees NSF's funding, to discuss his concerns. (The bill must be reconciled with a different version passed last month by the House of Representatives that scarcely mentions industry.)

Massey was vague, however, on just how the Senate language — and the new commission — would affect the agency's planning. Although the commission is intended to get input on the industrial research question from researchers and others outside the agency and its usual advisory bodies, it is not clear how those comments will shape the final strategic plan. Unlike the nearly finished plan of the National Institutes of Health (NIH), the NSF plan may not even emerge as a single document, Massey says. And despite the obvious similarities between NSF's planned open hearings and the five "town meetings" that NIH has held

around the country over the past year, Massey said that he was not influenced by the lessons that NIH learned in its own tumultuous planning process.

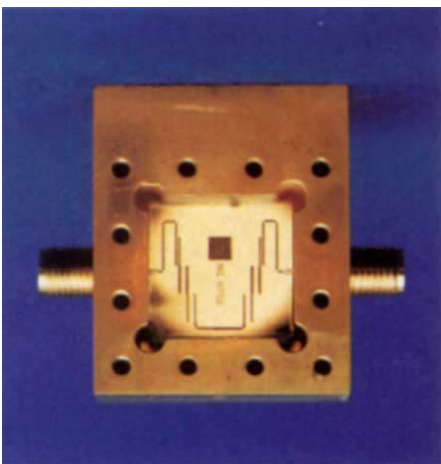
Instead, Massey said, the decision to turn to the scientific community at large reflects the magnitude of the change now foreseen for the agency. As well as increasing its support for industrial research, NSF is being asked to improve university buildings, undergraduate and pre-college education and US economic competitiveness. Given the degree to which such new responsibilities depart from NSF's traditional role of funding little else besides academic basic research, "we felt we needed a reality check" from the community at large, said James Duderstadt, president of the University of Michigan and chairman of the National Science Board, which endorsed the commission. The commission's members — who will come from universities, industry and other sectors — have not yet been named, but the panel is expected to hold at least three public hearings before it reports back at the end of the year.

Christopher Anderson

Superconductivity devices are headed into space

Washington. Only six years after the discovery of a new generation of high-temperature (high- T_c) superconductors, devices made from the materials are scheduled to be tested in space for the first time.

The US Naval Research Laboratory (NRL) will have the chance to launch two High Temperature Superconducting Space Experiments (HTSSE) — one in 1993 and the second in 1996. The goal is to “speed up the transition between basic research and system applications”, says George Price, project manager at NRL for the space experiments.



A space-bound, high- T_c filter

Each mission, costing \$20–\$25 million, will be launched into Earth orbit as part of the payload on a US military satellite. HTSSE 1 will carry 15 relatively simple devices, most of them filters used in communications satellites to select specific microwave frequencies from the incoming radio noise. The superconducting filters, on average, are ten times more discriminating than similar filters made of metal.

Researchers hope that the improved efficiency and lower energy losses from using high- T_c materials on satellites will lead to smaller yet more powerful computers, antennas and other subsystems. With weight at a premium on any launch, says James Ritter, who manages the NRL programme that is funding the experiments, “we’ve always believed that the key payoffs from superconductivity would come from space”.

Improved performance lies at the heart of the HTSSE programme. In January 1989, NRL sent out an invitation that, by the standards of the defence industry, was remarkably broad and open-ended. The agency was interested in funding, and flying, any kind of device that used the new high-

temperature superconducting materials. The only requirements were that the device should improve existing performance by at least an order of magnitude, and that it could be reproduced (NRL asked for five copies).

“We thought that was a tremendous challenge to industry”, says Ritter. Despite the fact that laboratories around the world were still discovering superconducting materials, the invitation attracted proposals from 35 organizations, including established companies such as Hughes Research Laboratory, American Telephone and Telegraph (AT&T) and Space Systems Loral and such start-ups as Superconducting Technology, Inc., of Santa Barbara, California, and the University of Wuppertal in Germany. All but two of the 15 devices on HTSSE 1 are made of YBCO (yttrium-barium-copper-oxide) superconducting thin films on various kinds of substrates.

The HTSSE 1 experiment will evaluate the performance of each device in space and how it is affected by radiation. A cooling system on board will maintain an operating temperature of 70–75K. HTSSE 2 will have more ambitious goals, including the testing of entire satellite subsystems that use superconducting components. It will also include communications tests from Earth to orbit.

Eventually, high- T_c superconductors may even make possible new kinds of spacecraft instruments, such as multispectral sensors that in a single device can detect radiation ranging from microwave to ultraviolet energies. Such a sensor could be placed on a space probe to the outer planets, where the ambient temperature is below the transition temperature of the new superconducting materials.

HTSSE is the US government’s most visible effort to apply the discovery of high-temperature superconducting materials, but research is also being sponsored by the Defense Advanced Research Projects Agency (DARPA), the Strategic Defense Initiative and elsewhere. DARPA alone will spend \$36 million this year on high- T_c research, only a fraction of which is related to space applications. Knowledge and hardware from these projects will increase the value of the NRL experiments “by at least a factor of five”, says Ritter.

Ritter hopes that successful HTSSE missions will lead to additional experiments. In the meantime, says David Chaffee of the trade newsletter *Superconductor Week*, HTSSE has given some focus to the budding superconducting market. “At a time when it seemed like things were going to be stuck in research forever”, says Chaffee, “HTSSE provides a means to look beyond that”.

Tony Reichhardt

NSF unmoved by politics of relocation

Washington. A forced shift towards supporting industrial research (see page 611) is not the only move the National Science Foundation (NSF) is contemplating. The site of the agency’s headquarters has become a political football, and a congressional committee has launched an investigation into the circumstances behind a decision to move the agency from the District of Columbia to a new building a few miles away in suburban Virginia.

For several years, NSF has been scheduled to move from its cramped and shabby — but well-situated — office building three blocks from the White House. Although many agency officials are reluctant to give up their proximity to power, a move to the suburbs appeared to be the only way to obtain badly needed additional space and modern administrative conveniences. As with every federal agency, NSF must do as it is told by the government’s landlord, the General Services Administration.

However, two states — Maryland and Virginia — surround Washington, and each wanted the agency as a tenant. After a long fight between politicians from both states, Virginia won and in 1990 the government signed an agreement to occupy a 450,000-square-foot facility in the Virginia suburb of Ballston in 1993. In addition to an atrium, the building features conference rooms and space for large meetings.

Maryland, however, never gave up, and NSF officials made it clear that they wanted to remain downtown. Earlier this month, Senator Barbara Mikulski (Democrat, Maryland), the chairwoman of the appropriations committee that funds NSF, stripped from the agency’s 1993 budget an amount roughly equal to the \$16 million that NSF intended to spend next year to move. The House bill explicitly removes any money for a move.

Concerned that politics — rather than NSF’s interests — may be becoming the focus of the debate, Representative Howard Wolpe (Democrat, Michigan) chairman of the investigations subcommittee of the House science committee, asked the congressional General Accounting Office (GAO) in June to weigh the pros and cons of the move and to investigate the way in which decisions have been reached. But now even that probe is feeling the political heat. Virginia’s two senators have asked GAO to rush the report and write up its findings in a letter rather than the usual more comprehensive report.

NSF, meanwhile, is lying low. Walter Massey, the agency’s director, said last week that plans are in abeyance, pending the completion of the GAO investigation.

Christopher Anderson

Korea begins funding projects to catch up with rest of world

Seoul. South Korea's Ministry of Science and Technology (MOST) last week announced the first projects in a \$6-billion effort by South Korea to catch up with the technology of Japan and leading Western nations by the beginning of the next century.

The Highly Advanced National Project,

than 2,400 billion won by 2001, matched by a similar contribution from industry.

Last week's announcement covered three of the 14 proposed research areas for the G-7 project: new pharmaceuticals and agrochemicals, new advanced materials and new functional biomaterials. These three

topics come under the jurisdiction of MOST. Other G-7 projects, covering such areas as high-definition television, semiconductors, integrated services and data networks and computerized manufacturing systems, will be announced shortly by other ministries.

More than 20 government institutes, 50 universities and 60 private companies will participate in the projects announced last week. Leading them on the government side is the Korea Institute of Science and Technology

(KIST) and the Korea Research Institute of Chemical Technology (KRICT). Major industrial participants include such conglomerates as Hyundai, Samsung and Daewoo and several pharmaceutical companies.

A total of 27 billion won (US\$34 million) has been set aside by government and

industry for the three research areas in 1992. Just over half of this budget was assigned last week (see table) with the rest to follow in a few months.

The lion's share of funding goes to development of new drugs and vaccines, in particular antibiotics. Dozens of pharmaceutical companies and several universities will participate in this effort, which is led by KIST (see sidebar) and KRICT. And two new nonprofit government-industry consortia — the New Medicine Development Consortium and the Genetic Engineering Research Consortium — have been established.

Companies such as Hyundai, Samsung and Daewoo are backing the projects to develop advanced materials, such as ceramics, for use in heavy industry, automobile manufacturing and the electronics industry. But so far there is no sign of participation by other nations, as Korean officials had hoped (see *Nature* 357, 100; 1992).

David Swinbanks

Where the money goes

(in millions of won, 790 won = US\$1)

	Government	Industry
New pharmaceuticals and agrochemicals	7,300	2,812
Antibiotics		
Herbicides/insecticides		
Hepatitis drugs/vaccine		
Anti-cancer drugs		
Anti-viral drugs		
Hypertension drugs		
Benzonoid germicide		
Other drugs and vaccines		
Screening of drugs and agrochemicals		
Common basic technology & international cooperation		
New advanced materials	2,959	1,544
High-strength aluminium		
Ceramics/ceramic engine		
Polymers		
Composite materials		
Heat- and wear-resistant materials		
High-density magnetic materials		
New functional biomaterials	132	132
Biodegradable polymer		
TOTAL	10,392	4,489

more popularly called the G-7 project, is intended to bring South Korea's technology up to a level competitive with the G-7 nations (Japan, United States, Canada, France, Germany, Italy and the United Kingdom) by 2000 (see *Nature* 354, 176; 1991). The Korean government intends to invest more

Defence scientist quits Indian post for US academic job

New Delhi & Washington. India's chief defence scientist for more than a decade, V. S. Arunachalam, has resigned in frustration over the lack of funding for major defence research and his government's continued reliance on foreign military technology. Arunachalam will be spending the next two years as a visiting professor at Carnegie Mellon University in Pittsburgh, Pennsylvania.

As head of the Defence Research Development Organization (DRDO), Arunachalam oversaw 35 defence laboratories with an annual budget of 11 billion rupees (US\$300 million) and is credited with the country's rapid progress in defence research. But Arunachalam was criticized for his agency's failure to develop a main battle tank, a project begun in 1975, and a light combat aircraft, begun in the early 1980s. In response, Arunachalam blames the government for failing to provide sustained support.

He also blames the government for being in such a hurry that it would rather import defence technology than wait for the homegrown product. As a result, he says, the DRDO has been hurt by "a great deal of invisible foreign lobbying" from companies peddling their wares to India.

But Arunachalam says these internal battles are not the reason behind his resignation, saying instead that he has long wanted "a change of pace". At Carnegie Mellon, he will work on the mechanical properties of materials and on technology transfer. He intends to take a new position with the Indian government when his sabbatical ends.

K.S. Jayaraman & Traci Watson

G-7 is wake-up call for KIST

Seoul. Jung Uck Seo, the newly appointed president of the Korea Institute of Science and Technology (KIST), says that the G-7 project and others like it are intended to wake up his institute from the "dreamland of the 1970s" and bring his researchers closer to addressing the needs of society. KIST, based in Seoul, is one of South Korea's leading research institutes with more than 750 researchers, including those in affiliated institutes in Daeduck science city south of the capital.

The institute was established by the Ministry of Science and Technology in 1966 to bring together academic institutions and industry. But Seo and other government science policy-makers feel that some KIST researchers have lost touch with the needs of industry, and that G-7 will bring them back into line.

KIST was modelled on national research laboratories in the United States, and despite its location in overcrowded Seoul boasts a large campus. Seo says that KIST must now serve a future based on Korean culture; it was a "baby delivered without pain", he says, but after 25 years it has "reached adulthood".

As an example, he points to South Korea's development of digital switching systems for the telecommunications industry. "If we had not made the effort to develop those switches ourselves, our telecommunications networks would have been completely dominated by one US company", he says. "Instead, now we export our switches." **D.S.**

Bovine growth hormone meets new safety concerns

Washington. The controversy surrounding recombinant bovine growth hormone (rbGH), a genetically engineered animal drug, surfaced again last week after a government report suggested further evaluation of its risk to humans.

The report* by the US General Accounting Office (GAO) recommended that the US Food and Drug Administration (FDA) withhold its approval until more is known about its indirect impact on food from cows that have been given the drug. The GAO's findings could represent a considerable setback for the four companies — American Cyanamid Company, Elanco (a division of Eli Lilly & Company), Monsanto Company and Upjohn Company — seeking approval from FDA to market their versions of rbGH in the United States. The drug can be used to boost milk production in dairy cows by 10–25 per cent.

The report concludes that even though FDA has been aware for some time that rbGH-treated cows have a higher incidence of mastitis (inflammation of the udder), the

agency has failed to gather the data to determine whether this would result in an increase in the use of antibiotics and therefore higher antibiotic residues in meat and milk — so-called 'indirect' health effects. A congressional aide to US Representative Ted Weiss (Democrat, New York), one of nine congressmen to request that GAO evaluate the thoroughness of FDA's investigational review process for rbGH, called this a "serious shortcoming" in the review process.

GAO went on to recommend that until FDA has evaluated these indirect human food safety risks, the agency should suspend the sale of meat and milk products from rbGH-treated cows. FDA approved the commercial sale of meat and milk from experimental herds in late 1985 after deciding it was safe for human consumption.

Because the report was more than two years in the making, it says little that is new to the FDA, says Gerald Guest, director of the FDA's Center for Veterinary Medicine — the division of FDA that will decide whether or not to approve rbGH for use in

dairy cows. The problem of indirect health effects is still an "open question", says Guest. "It's one that has arisen before and that will be part of what has to be resolved before the drug can be approved", he says.

Guest says that he is not concerned about the safety of milk from the 700 or so cows now being treated with rbGH because it is so carefully regulated. "The real question is, if you were to approve it [rbGH] and it becomes widely used in a less-controlled fashion, would there then be a problem?"

Predictably, news of GAO's findings was welcomed by Jeremy Rifkin's consumer watchdog group, the Foundation on Economic Trends. The group, which has led a seven-year campaign to keep rbGH off the market citing concerns for human and animal health, filed a legal petition with FDA last week calling for full compliance with GAO's recommendations.

In a separate but related GAO report†, FDA was criticized once again for its failure to develop a comprehensive strategy for monitoring animal drug residues in milk. The report highlighted the large gap between the number of drugs used to treat dairy cows, which may leave residues in milk, and the number of drugs that are routinely tested for at the state level and by FDA.

Diane Gershon

* Recombinant Bovine Growth Hormone: FDA Approval Should Be Withheld Until the Mastitis Issue is Resolved (GAO/PEMD 92-26, 6 Aug 1992)

† Food Safety and Quality: FDA Strategy Needed to Address Animal Drug Residues in Milk (GAO/RCED 92-209, 5 Aug 1992)

Dutch flower may be too safe to test new biotechnology laws

London. A white chrysanthemum is destined to become the first test of European legislation on the commercialization of genetically modified organisms when it is submitted to the Dutch authorities later this month. But the flower, called Floriant, may be too ordinary to serve as a good test case.

Originally pink, the chrysanthemum has been altered with DNA from another strain so that the flowers are white. Florigene, the company concerned, feels that using such a 'neutral' product to test the system will make progress easier. Although it is not expecting a great commercial return from Floriant, the company hopes that the flower will pave the way for other products being developed. While still in the realm of cut and ornamental flowers, these products will have improved resistance to diseases and pests.

Although recognizing the value of testing the system, others in the biotechnology industry do not understand why Florigene is using a product that is so simple and is not necessarily a commercial priority. Don Emlay, director of regulatory affairs for Calgene, a California biotechnology company, says that he doubts whether the knowledge gained would offset the cost in time, effort and people involved. The test comes when most of the countries of the European

Communities have at last introduced legislation to meet two directives on the subject passed by the European Council of Environment Ministers in April 1990.

"There are other ways of interacting with regulatory agencies that can lead to a better understanding without having to go through a test case," he says. Calgene has recently applied to the US Food and Drug Administration and Department of Agriculture for permission to grow and market its Flavr Savr rot-resistant tomato (see *Nature* 357, 352; 1992).

Unlike Europe, the United States has recently instituted regulations to cover genetically altered foods, and Emlay feels that Flavr Savr's advantage as a test case lies in the problems it raises. "Concerns for safety are greater with fresh foods than with foods that will be processed or non-foods" he said. "The tomato is a very good first product because it raises all the issues up front."

Contained trials and field trials of genetically modified organisms have been conducted in Europe and the United States for some years. However, those trials are usually carried out with such precautions as netting and barrier plants that lessen the chance of genetically altered material spreading beyond the test field.

Ian Mundell

University scientists to be investigated for conflicts of interest

Washington. A powerful congressman has turned his investigative team loose on Stanford University and the University of California after receiving reports from a whistleblower of potential conflicts of interest among university scientists, according to a report last week in the San Jose (California) *Mercury News*. Following a tip from Paul Biddle, the former government accountant who initiated the 1989 indirect cost investigation at Stanford, the staff of Representative John Dingell (Democrat, Michigan) are investigating scientists at the two universities who may have used the results of government-funded experiments to benefit private companies or have channelled federal grant dollars to private research. Dingell's oversight and investigations subcommittee of the House Energy and Commerce Committee has tentatively planned a hearing for late September. According to the news report, the subcommittee intends to widen the investigation to universities across the United States. A subcommittee staff member confirmed that an investigation is under way. **Traci Watson**

US government asked to fund more industrial research

Washington. In a move certain to fuel the debate over industrial policy, the US National Science Board released a report* last week that laments the amount of US industrial research and calls for increased federal

the government reorientate its research and development (R&D) budgets away from defence missions and towards the needs of industry. And it calls on federal science agencies to give greater consideration in awarding grants to US industrial competitiveness or technologies in which the United States is slipping.

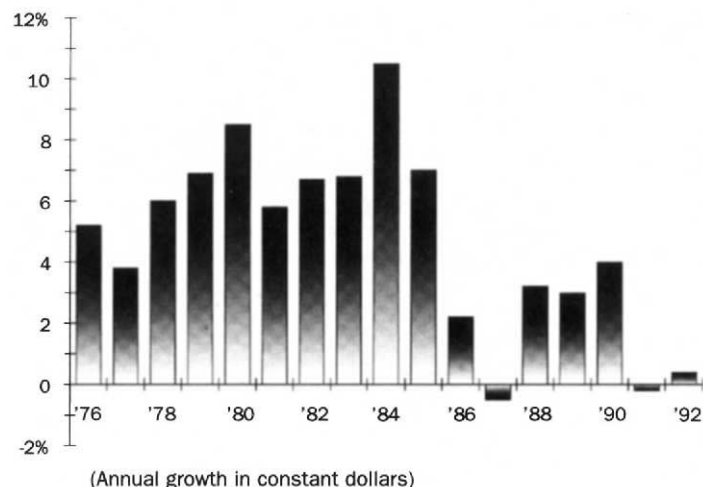
The board calculated that the United States would now have to increase its R&D spending by \$25 billion (about 16 per cent beyond current levels) to match Japan, the world's leader in industrial R&D spending relative to GDP. It

blames the gap on a preoccupation within industry on short-term investment and corporate restructuring. But it also calls on Congress and the administration to make it easier for industry to increase its domestic research spending by making permanent a now-temporary R&D tax credit and by removing a portion of the tax code that encourages US companies to move their R&D to foreign countries to receive a tax break on overseas sales.

Christopher Anderson

* *The Competitive Strength of US Industrial Science and Technology: Strategic Issues*; National Science Board, 1992

Hard times for industrial R&D



support of industry. Citing a number of recent studies and some previously unpublished surveys that show a stagnation in US industrial research spending relative to gross domestic product (GDP), the board recommends that the government "re-examine [its] traditional isolation from business issues" and increase its support of programmes that directly help industrial product development and manufacturing.

The board, the policy arm of the National Science Foundation, did not actually use the words "industrial policy", a term that is still anathema to the administration. But it clearly endorsed the concept. It recommends that

Government spending on R&D (1989 percentage)

	US	Japan	Germany	France	UK
Defence	65.5	9.0	19.0	41.9	55.2
Civil space	7.3	11.1	8.5	8.7	3.8
Advancement of research	3.8	13.8	20.7	17.5	5.8
Health	12.9	4.8	5.2	3.7	6.2
Industrial development	0.2	8.1	19.0	15.0	10.3
Energy	3.9	39.2	9.5	4.0	4.0
Agriculture, forestry and fisheries	1.9	6.5	3.1	4.6	5.5
Other	4.5	7.6	14.9	4.5	9.2

NEWS IN BRIEF

Washington. US legislators passed a bill last week that will explicitly define crimes committed at animal research facilities as federal offences. But because backers have been forced to weaken the bill considerably in their three-year struggle to pass such animal legislation, its effect will be mostly symbolic. It covers only crimes that are committed on an interstate basis and cause damage exceeding \$10,000 — conditions that would make the crimes federal offences regardless of any special law. However, the bill extends federal law by including a provision requiring those found guilty to pay the cost of repeating disrupted experiments. It also requires a federal study on the effect of terrorism on animal facilities, which include agricultural enterprises as well as research institutions. **C.A.**

Washington. An animal-rights magazine revealed last week that one of its own — the US National Anti-Vivisection Society (NAVS) — held \$88,000 of stock in US Surgical, a medical supply company that has become one of the chief targets of animal activists. *The Animals' Agenda* reported in its July/August issue that NAVS also holds stock in Wal-Mart Stores, which have been boycotted by animal groups for selling live animals to "puppy mills". Mary Margaret Cuniff, the society's executive director, explains that the US Surgical stocks were purchased last year without the organization's knowledge by a money manager, and that NAVS divested itself of the stock earlier this year when it discovered the mistake. "Of all the companies [the manager] could have purchased, US Surgical was the worst", she says. NAVS received a \$2-million bequest several years ago and now uses independent consultants to manage its financial policy to decide how to bring its investments in line with its ethics. **C.A.**

Washington. A bill to create a science foundation to promote research in former Soviet states has passed both houses of Congress and needs only a budget before it can be put in place. Its sponsors have requested \$25 million, but its fate is tied to the resolution of the 1993 defence appropriations bill, from which the foundation would be funded. The bill, a brainchild of Representative George Brown (Democrat, California), directs the National Science Foundation (NSF) to establish a non-profit, non-government foundation to foster basic research and technology transfer in the former Soviet states (see *Nature* **355**, 576; 1992). The NSF is expected to model the foundation after a similar US-funded organization in Israel supporting Israeli science. Once funded, the new foundation is expected to award money to US-Soviet research teams on the basis of peer-reviewed grant applications and to encourage the states' academic scientists to collaborate with industry on non-defence research and development. **T.W.**

Frustrated by regulatory delays at FDA, biotech companies warm to user fees

Washington. The US biotechnology industry has reversed its policy and now accepts user fees as the best way to win rapid approval of new products by the US Food and Drug Administration (FDA). At a congressional hearing last week, industry representatives testified in support of FDA's plans to levy fees so that it could hire some 600 scientists and hasten the review of new drug applications. Although industry dislikes the concept, many companies have come to believe that industry subsidies are the only way to speed the process at a time when major funding increases for FDA are unlikely.

David Kessler, the FDA commissioner, testified last week that user fees could provide a financial lift to an agency faced with an expanding work load, particularly in biotechnology. A bill containing the provision is expected to be introduced next month in the House of Representatives by Henry Waxman (Democrat, California) and John Dingell (Democrat, Michigan).

This belated endorsement of user fees by industry is "recognition of the gravity of the situation and the necessity for action", says Richard Godown, president of the Industrial Biotechnology Association (IBA). He points out that it is the first time there has been agreement between the legislative branch, the regulatory branch and the industry on the issue. As recently as January, when President George Bush presented Congress with his budget request for fiscal year 1993, IBA opposed the administration's proposal for user fees out of fear that it would increase the already high costs of drug development and hinder product research and development. Industry executives have argued that user fees would be particularly burdensome to the smaller biotechnology companies with little or no sales revenues.

At a press meeting last month, Kessler said that the levying of user fees had become "absolutely critical". Without them, he said, "this agency is not going to be able to survive". Agency officials were forced to institute a hiring freeze in July after it became clear that FDA would receive little more than a cost-of-living increase for next year. Last week, Congress confirmed FDA's fears and gave the agency \$746 million, only 2.8 per cent more than this year's budget.

Kessler says that he would use the money raised through user fees to hire an additional 300 inspectors for the Center for Drug Evaluation Research (CDER) and another 300 for the Center for Biologics Evaluation Research (CBER). In return, Kessler says that FDA would aim to shorten to six

months the average review time for priority drug applications, that is, drugs for life-threatening diseases or diseases for which there are no alternative therapies. Review times for other standard drug applications would be reduced by half from an average of two years to one year, according to the FDA.

FDA hopes to be able to raise \$35 million in user fees in the first year, \$50 million in the second and \$75 million in the third and each year thereafter. Companies would typically make a one-time payment of about \$150,000 for drug applications and pay smaller annual fees for products that are on the market and for manufacturing plants. Smaller companies may be charged lower fees for some services. Initially, neither medical devices nor generic drugs would be included in the plan.

While the three trade associations — IBA, the Association of Biotechnology Companies and the Pharmaceutical Manufacturers Association — have agreed in principle to the concept of user fees, many

details are not yet resolved. Industry would like to see the fees used to improve the speed and quality of the review of new product applications (that is, new drug applications and product licence applications) carried out by CDER and CBER rather than for such FDA activities as enforcement. In addition, the industry wants the FDA to accept 'performance standards' that, if not reached, would allow Congress to refuse to reauthorize its programmes.

Larry Kurtz, vice-president of corporate communications for Chiron Corporation in California, says his company supports the concept of user fees so long as they are "an increment to the FDA budget and not simply a substitute for reduced federal spending".

Waxman and Dingell will be hard-pressed to get the bill passed in the few months that remain in this current legislative session. But the bill is expected to move quickly next spring once it is brought before a new Congress.

Diane Gershon

Scientists deny alleged support of company's 'new nuclear science'

Washington. Ten scientists cited as supporters of a nuclear theory that is the foundation of a new cold fusion company are complaining that they do not in fact support the theory and that their names have been used without their knowledge.

A little-known physicist-entrepreneur, Ronald Brightsen, claims to have pioneered a new nuclear science based on his 'nucleon cluster model' of the atomic nucleus. In a press release dated 10 August, Brightsen asserts that the model will "provide a clear path" to "cold fusion power", and lists 16 'Supporters of the Nucleon Cluster Model', many of them scientists. But the ten who could be reached last week deny supporting Brightsen's model and object to the use of their names.

Brightsen's new physics rests on the premise that the atomic nucleus consists of discrete 'clusters', each containing a different number of neutrons and protons. He has predicted that cold fusion home heaters based on this theory could be on the market within three to ten years. Brightsen, who earned a master's degree in nuclear chemistry from the Massachusetts Institute of Technology (MIT) in 1950, claims his findings have been "praised by leading experts" on nuclear energy.

But many physicists, including some of Brightsen's alleged supporters, have dis-

missed his model. "When I [last] saw it several years ago, it was more numerology than anything else", says Warren Buck, a professor of physics at Hampton (Virginia) University who is listed as a supporter.

Buck, like many of the other scientists listed in the press release, says that he reviewed the cluster model during the 1980s at Brightsen's request and found it interesting but unconvincing. Besides Buck, ten other 'supporters' have repudiated the model. (Of the remaining five, two could not be contacted and two — including retired Admiral Elmo Zumwalt, the former chief of US naval operations — are non-scientists.)

To develop cold fusion and other technologies based on his findings, Brightsen and others have founded Clustron Sciences Corporation (CSC) in Reston, Virginia. CSC's vice president of research is Eugene Mallove, a former MIT science writer who accused researchers at the MIT Plasma Fusion Center of misconduct for suppressing possible evidence of cold fusion (see *Nature* 353, 98, 1991).

"Perhaps 'supporters' was a bad choice" of words, Brightsen admits. But he says "we think what's in the press release is accurate, subject to interpretation."

Traci Watson

Offspring of scientists

SIR — R. C. Connolly¹ comments that the sex ratio (proportion of the total population who are male) of 0.5278 among the offspring of artists² may be linked to the size of the artists' families. Reviewing the data, I find an average of 2.333 children per artist in the group (of 1,489 artists), and indeed Connolly, judging from the sex ratio, predicted that the average might be between two and three. But family size considerations appear unable to account for the very low sex ratio of 0.4956 among the offspring of 1,882 scientists and mathematicians, where the average number of children is 2.742; Connolly quoted sex ratios of 0.5294 and 0.5271 for families of two and three children respectively, for the United Kingdom generally. The 0.4956 sex ratio for the scientists' and mathematicians' families differs significantly ($P > 0.001$) from even the lower of the above two values given by Connolly. There remains a possibility of a link between parentage and offspring sex ratio. Studies such as that of Crawford *et al.*³ are of considerable value in this context.

R. A. Beck

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R. C. CONNOLLY REPLIES — R. A. Beck's further analysis of his data on the offspring of artists¹ is to be welcomed, not least because it accords with my suggestion of probable family size in his cohort² but also because it contributes yet more information to our extensive but incomplete understanding of the control of the sex proportion in human populations.

His new data (see above) on sex proportions of scientists and mathematicians in 1882 must be reviewed in the light of population statistics at that time rather than directly with my figures which are secondary sex proportions (at birth) from 1956 onwards. As has been noted⁴, the sex-proportion at birth is not a stable value and postnatal death rates which would be reflected in the quoted family sizes also show marked secular variation⁵.

The population structure of the relevant years for England and Wales around Beck's observations derived from tables published by the Registrar General and reviewed by Britton and Edison⁵ and Hocking⁶ give birth rates with the following sex-proportions:

1871—75	0.5096
1876—80	0.5093
1881—85	0.5093
1886—90	0.5088

Values for living children in the population in 1881 at different ages are

as follows:

0—4yr	0.4992
5—9yr	0.4985
10—14yr	0.5007
15—19yr	0.4980

I am not aware of published data for these periods relating sex-proportions to birth order which would complete the figure and allow a valid comparison for Beck's data on scientists and mathematicians but the sex-proportions quoted above for whole families do approach Beck's cohort value of 0.4954 without knowing family size.

It is very likely that there is a link between parental genetics and sex-proportions but the extent of this contribution is still inadequately understood and sadly, for populations in the last century, the census figures may be less accurate and thus less representative of the population as a whole than the results of more recent surveys.

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□ This correspondence is now closed. — Editor, *Nature*.

NIH patent rights

SIR — The pending patent application filed by the National Institutes of Health (NIH) on 2,421 brain cDNA clones obtained by J. Craig Venter and colleagues (so-called Expressed Sequence Tags) constitutes a body of intellectual property owned by the US government. While the wisdom of the NIH filing of this patent, now a continuation-in-part application, is a matter of legitimate controversy, the deed is done. If some or all of the patent claims covering uses of the cDNA clones (for example, for the identification of human brain tissue, the creation of probes, antisense oligonucleotides and vectors, and mapping the expressed sequences on chromosomes) are eventually allowed by the US Patent and Trademark Office, which is by no means certain, the NIH will be entitled to receive licence fees and product royalties from companies that make use of the claimed subject matter.

Venter's new undertaking is described as a "nonprofit research centre" but its investors are also planning a new biotech

company to develop and market products from this technology (see *Nature* **358**, 95; 1992). We trust that any patent rights will not be given freely to a single company for the commercial benefit of a few investors.

If patents are issued, it is to be hoped that the NIH will negotiate appropriate licence fees and royalties from all commercial users, so that this taxpayer-funded research will generate capital to further the endeavours of one of the world's greatest nonprofit research centres, the NIH.

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Leeds inquiry

SIR — In his response (*Nature* **358**, 102; 1992) to your leading article about allegations against me of scientific misconduct, J. J. Walsh, the Registrar of the University of Leeds, claims that his inquiry "followed guidelines modelled on those recommended by the Royal College of Physicians". These call for thorough investigation of all related matters and for protection of the complainant. I had emphasized that I was more concerned in the subsequent events and in particular with attacks on my post which I believed were related to my discovery of the fraud. It is most regrettable that the university constructed a remit for inquiry which seemed to have excluded the very matters with which I was most concerned.

I have no doubt a truly independent investigation of the evidence I presented would arrive at substantially different conclusions from those of the Leeds panel. If Walsh is convinced that the investigation by the University of Leeds did not avoid the main issues, and that senior university staff had no significant role in the steps leading to my dismissal, then he should welcome confirmation of their findings by truly independent investigators. I hope he will accept this challenge.

For the record, Walsh refused all my requests for investigation until *Nature* indicated an interest in my case. When the "three wise men" inquiry was announced, the vast majority of people thought a "cover-up" to be the most likely consequence. Rightly or wrongly, this view of self-policing by universities appears to be universal. I am offering the University of Leeds an opportunity to demonstrate that this cynicism is unjustified.

Chris Chapman

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Defining consciousness

SIR — We are pleased to learn¹ that the convention that 'consciousness is so ill-defined that it lies beyond the reach of serious study' is losing ground. Something is still missing from the studies reported by Jeffrey Gray, however, especially an acknowledgement of the fact that consciousness has more subtle aspects than examples such as 'red', 'pain' and 'itch', and more importantly that it can be studied from within.

We know, for example, about belief and hope because we believe and hope ourselves, and are aware of the fact that we do so. Such attributions are traditionally called by psychologists 'fictions' (as opposed to the 'facts' discovered by science).

But this fact/fiction duality is a false one²: modern physics, for example, posits many entities that through their unobservability may be considered just as much fiction as fact, and hopes and beliefs are just as much fact as fiction.

The same, we believe, can be said of more delicate aspects of experience such as those pertaining to the arts, or spiritual experience. Such experiences, to the extent that they are capable (as is frequently the case) of being put into words understood by others, can become knowledge and understanding that, like scientific knowledge, can be discussed in depth and become ultimately the shared, usable knowledge of an expert community.

Science's insistence upon the fact/fiction dichotomy referred to (in common with other ultimately spurious distinctions, such as the supposed sharp distinction between science and philosophy) is the outcome of the way scientists are trained to think: to see only certain forms of observation, certain forms of argument and certain categories of description as being 'correct'.

Such constraints, while indispensable as a tool for carrying out science as at present conceived, create a limited perspective upon reality as a whole, denying, for example, the real existence of meaning and value, and (assuming that the following fiction-word has meaning!) devaluing introspective investigations into (to take a particular example) the problem of personal identity ('who — or what — am I?')^{3,4}. The question 'what is it like to be a human being?' should, for human beings, be easier to answer, and more relevant to human needs, than Nagel's question 'what is it like to be a bat?'

What we have argued about entails what we see as a necessary revision in the way the problem of consciousness is viewed, involving the adoption of a vantage point from which knowledge is

viewed as a unified whole, of which scientific knowledge is but one aspect.

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SIR — Jeffrey Gray's fascinating article ends on a tantalizing note. One needs more than the gathering of experimental data, he concludes — one needs "... a new theory ... that will render the relations between brain events and conscious experience 'transparent' ... This new theory is at present unimaginable ...".

Such a theory may indeed need to be profoundly different from any existing one if it is to transcend the limitations of neurocomputational models. But just such a theory has been imagined, and with great force and originality, by Gerald Edelman, and has been set out in detail by him in several books (most especially in *The Remembered Present: A Biological Theory of Consciousness*). Whether Edelman's full theory of neuronal group selection will be confirmed by rigorous testing is as yet unclear; but it represents a monumental theoretical construct, and surely deserves a mention.

Oliver Sacks

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Frontiers future

SIR — As one of the founders of the Human Frontier Science Program, I should like to comment on the present controversy over the future of the programme (see *Nature* **358**, 527; 1992).

(1) Human Frontiers puts a relatively

small amount of money into two areas of research: brain functions, and 'molecular approaches' to elucidation of biological functions. The money spreads to countries that already have well-funded research programmes in traditional areas of biological research. Frontiers should not seek to compete with these existing programmes. Rather, through its uniqueness it should seek to add an international component to research.

(2) Frontiers supports two selected areas, but advances in these fields will come from other areas, in particular chemistry, physics and computational science, which offer not just new techniques, but also new concepts and different approaches. The programme should foster growth of interdisciplinary research at the borders of biology.

(3) The secretary general of the programme should have a wide knowledge of science and the ability to move the programme into these areas. It is not a major administrative job, but one that requires intellectual leadership; it could be carried out on a part-time basis.

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Motes and beams

SIR — I agree that "there must, after all, be some standards" (*Nature* **358**, 2; 1992) regarding the use of English in scientific publications. Having published a news item in which "*bacillus thuringiensis* (Bt)" [lower case for the genus name, no italics for the abbreviation] is described as "a bacteria" (*Nature* **355**, 661; 1992) *Nature* is in no position to put itself on a pedestal as the guardian of those standards.

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Dam facts

SIR — You say (*Nature* **356**, 363; 1992), "Who but the US Army Corps of Engineers built Boulder Dam?"

The answer is that it was constructed by the Six Companies, Inc. for the US Bureau of Reclamation, which engineered it and owns it.

Bryant Mather

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The mysterious virus called "isn't"

The first close scrutiny of reports of a new virus supposed to cause symptoms of AIDS in people with neither HIV-1 nor HIV-2 suggests that there is no new virus.

CAN one have AIDS without the AIDS virus? Or is it possible that a third AIDS virus (neither HIV-1, the commonest form of the virus in the United States and Europe, nor HIV-2, which appears to be endemic in Africa) is lurking somewhere in the tissues of immunosuppressed individuals who have precariously low concentrations of CD4 T cells — the lymphocytes whose progressive depletion is associated with the development of AIDS?

Worldwide concern over the possibility that a previously unidentified infectious AIDS-like virus may be causing severe immunodeficiency disease was ignited last month by an article in *Newsweek* that asked, "Is a New AIDS Virus Emerging? The patients are sick or dying and most have risk factors. What they don't have is HIV."

Last week, researchers from the US National Institutes of Health (NIH) and Centers for Disease Control (CDC), together with AIDS and infectious disease experts from institutions all over the United States, held a one-day conference at CDC in Atlanta to assess data from several laboratories reporting retroviral activity in the cells of patients who appear not to be infected by HIV.

They reached a consensus on several points.

■ First, as Anthony S. Fauci, AIDS coordinator for NIH put it, "It is essential that we keep an open mind."

■ Second, present data do not support the suggestion that there is a third virus, which is why some are calling it the "isn't" virus. (The mystery virus has also been dubbed "MTV" for media transforming virus in the light of all the press attention it has received.)

■ Third, there is even less evidence that the alleged virus or virus particles, were they to be confirmed, cause disease.

■ Fourth (and most important), members of two expert panels — one to evaluate epidemiological data, the other retrovirology — find nothing to suggest they are dealing with a new transmissible agent. This point is central to the issue underlying current anxiety — namely, the safety of the blood supply. The analogy has been made to the early days of the AIDS epidemic when scientists were uncertain what they were dealing with, but the analogy does not hold. From the outset, AIDS cases appeared in clusters, particularly in California and New York, and there was evidence that physicians were dealing with an infectious disease. But that is not the case here.

The *Newsweek* article, faxed to reporters

in Amsterdam where the international AIDS meeting was in full swing during the week of 20 July, based its arresting question on the work of a couple of research teams who have reported CD4 patients. One team is that of Jeffrey Laurence of Cornell University Medical College in New York, who reported in the 1 August issue of *The Lancet* (340, 273; 1992) on five patients (four of them at risk for AIDS) who have low counts of CD4 T cells, various opportunistic infections and no apparent signs of HIV-1 or HIV-2 in the blood. In the *Lancet* article, not published until after *Newsweek* hit the faxes, Laurence suggests that the cases he describes "raise the question of the existence of other agents associated with transmissible immune deficiencies that can evade current laboratory detection techniques".

One of Laurence's co-authors, Stephen Morse of Rockefeller University, acknowledges that they may be seeing an artefact, a contaminant or viral activity from an endogenous retrovirus. But, he says, the issue is still "open" and research will proceed. "Our working hypothesis is still to look for a virus." Data similar to those from Laurence's laboratory were presented in Atlanta by David Ho of the Aaron Diamond AIDS Center in New York. He has seen more than a dozen patients, several of whom do not have the usual risk factors associated with AIDS — drug use, a history of blood transfusions or multiple homosexual partners. Two of them have signs of reverse transcriptase activity, but none is seropositive for HIV. Ho sent his material to an outside laboratory expert in retrovirus detection, but the answer came back negative.

The data that have probably received the lion's share of press attention were reported by Sudhir Gupta and colleagues at the University of California at Irvine. In a paper in the 15 August issue of the *Proceedings of the National Academy of Sciences* (89, 7831–7835; 1992), which was released early in response to the public furore in Amsterdam, Gupta reports detection of a human intracisternal retroviral particle associated with CD4 T-cell deficiency in a 66-year-old woman with *P. carinii pneumonia* and her healthy, but CD4-depleted, daughter. (Gupta and the university have applied for a patent on the particle.)

Gupta's data are viewed with considerable scepticism by retrovirologists, several of whom have summed up their evaluations by saying, "there is nothing significant there".

Retroviral particles (particularly those known as A-type) have been studied on and

off for years, in animal and human systems, without evidence that they cause disease. In his paper Gupta reports that the particle he found (and which he calls both a particle and a virus) is distinct from A-type particles. His particles do have *gag* and *pol* genes as HIV does, but lack the envelope gene that would allow the particles to get out of a cell. So the probability that they are infectious is said to be close to nil.

A fourth report at the Atlanta meeting by Robert F. Garry of Tulane University School of Medicine recapitulated two-year old data on retroviral particles and Sjögren's syndrome — an autoimmune disease that causes dryness of the eyes and mouth. In a paper in *Science* (250, 1127–1129; 1990) Garry and his colleagues reported a human intracisternal A-type retroviral particle in Sjögren's patients that is antigenically related to HIV but is distinguishable from it by ultrastructural and enzymatic analysis. Garry concludes not that the A-type particle causes Sjögren's syndrome but that the data support "an association between retrovirus infections and autoimmune phenomena that has long been suspected." An association, as yet unexplained etiologically or mechanistically (and probably unrelated to AIDS), may be what is emerging from the current reports of CD4-depleted patients.

So, where do things now stand? According to Fauci, there is general agreement that together Ho, Laurence, Gupta and others who have CD4-deficient patients may have identified a new and heterogeneous syndrome. "It could be decades old", he says. "It could just be coming to light because, for the past five years or so, we've been testing for CD4. We just don't know yet." CDC is calling the new syndrome ICL for idiopathic CD4 lymphopenia and there seems to be a consensus that it is epidemiologically and clinically real. The virology is more of a mystery but, Fauci says, the scepticism that greeted early reports in Amsterdam of a new virus "has really been reinforced by our analysis so far".

But good science and good public policy demand that the questions be more clearly resolved. NIH and CDC have established disease registries for CD4-deficient patients whose immune deficiency cannot be explained.

NIH and CDC have also established a repository for blood and other tissues from CD4-depleted patients that can be distributed to laboratories around the world for studies that may help to resolve this intriguing mystery.

Barbara J. Culliton

The tell-tail trigger

Michael Gregory Peterson and Robert Tjian

WHAT is the trigger that causes RNA polymerase II (Pol II), the enzyme responsible for transcribing protein-encoding genes, to initiate RNA synthesis once it has assembled into a stable complex at the promoter? This has been a sticky issue in the study of eukaryotic transcription, but one which has now become more tractable. In a paper on page 641 of this issue¹, and in others published or to appear elsewhere²⁻⁴, a critical component of the basal transcriptional initiation machinery is reported to be a kinase that specifically phosphorylates Pol II; the kinase may thus provide the signal to switch the enzyme from initiation into the elongation mode.

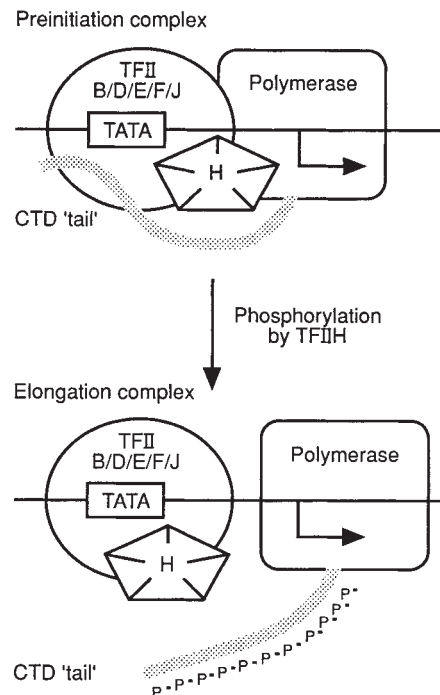
The fruit of a decade of frenetic investigation has been identification of a plethora of proteins (transcription factors) that are in some way involved in recruiting Pol II to the promoter and signalling the onset of transcription. Although many of these factors have now been purified, and the genes encoding them cloned, surprisingly little is known of their precise role in the initiation process. Accurate Pol II transcription can be reconstituted using highly purified components which are required at most if not all Pol II promoters. These general transcription factors (TFIIB, -D, -E, -F, -H and -J) are assembled in an ordered fashion at the promoter to form a stable preinitiation complex (see figure).

The first component recruited to the promoter is TFIID, itself a large multiprotein complex which binds to the TATA box by way of a critical subunit, the TATA box binding protein (TBP). Once TBP is tethered to the DNA, TFIIB can then enter to form the so-called DB complex. This provides an inviting landing site for Pol II which, in turn, is escorted to the promoter by TFIIF. Even though at this stage Pol II is positioned at the promoter, initiation cannot proceed without the orderly arrival of three additional factors, TFIIE, -H and -J.

What, then, are the functions of these ancillary factors, and how do they communicate with Pol II, the engine that drives RNA synthesis? The first clue to an answer came with the observation that Pol II comes in two forms, which differ only by extensive phosphorylation of the carboxy-terminal domain (CTD) 'tail' of the largest subunit of the enzyme^{5,6}. Intriguingly, the non-phosphorylated IIA form of Pol II is the one recruited to the promoter as part of the preinitiation complex, whereas the phosphorylated form, IIO, is found in

the elongation phase of the reaction. Evidently, at some stage between initial promoter binding and the formation of the first phosphodiester bond, the enzyme is converted to a highly phosphorylated form.

This phosphorylation event presents an attractive mechanism for triggering the initiation process. If the phosphory-



Model showing the action of transcription factor IIF (H) kinase on the carboxy-terminal domain (CTD) 'tail' of RNA polymerase II. *Top*, The general transcription factors, (TFIIB/D/E/F/J and H), are assembled into a preinitiation complex at a promoter consisting of a TATA box element and transcriptional start site (arrow). *Bottom*, Transcription factor IIF phosphorylates (P-) the CTD 'tail' of polymerase, uncoupling the complex and allowing elongation to proceed.

lation of the CTD were, in fact, an important step during initiation, one or more of the auxiliary transcription factors might be expected to catalyse the conversion of IIA to IIO. Thus, an intensive search for the CTD kinase was initiated. In mammals, the CTD consists of 52 repeats of the heptapeptide YSPTSPS (single-letter amino-acid code), and many kinases have been described that will phosphorylate it — which is not surprising, given its content of serine and threonine residues, and the promiscuity of most kinases *in vitro*. But phosphorylation of the CTD still occurs, even in the most purified reconstituted transcription systems available, suggesting that one of the general transcription

factors may actually be a CTD kinase.

That, it turns out, is indeed the case. Lu *et al.*¹, as well as Feaver *et al.*², Gileadi *et al.*³ and Serizawa *et al.*⁴, describe the purification and characterization of a CTD kinase activity that copurifies with one of the essential basal transcription factors, TFIIF. The strongest evidence that the kinase is indeed an integral component of this basal factor, and not a contaminating activity, comes from studies with a monoclonal antibody directed against the cloned 62K subunit of TFIIF⁷ which inhibits both transcription and the CTD kinase activity.

TFIIF has many of the desired properties for a kinase responsible for phosphorylating Pol II and initiating RNA synthesis. It seems that the kinase is held in check until the preinitiation complex is formed on the promoter. This is a telling point, which strongly implies that this kinase has a functional role linked to transcription. The data also show that its activity is increased in concert with the assembly of the preinitiation complex, reaching a crescendo upon addition of TFIIE, which apparently serves to enhance further the activity of the TFIIF kinase. Under these conditions, the kinase is proposed to move along the CTD, efficiently converting the IIA to the IIO form. Thus, a distinctive property of this kinase is its intimate and specific association with a functional initiation complex. The observation that the dephosphorylated, but not the phosphorylated, form of the CTD interacts with TBP suggests one possible mechanism for unhitching Pol II from the preinitiation complex⁷. Phosphorylation of the CTD by TFIIF may therefore provide a critical step in uncoupling the RNA polymerase from the preinitiation complex⁷ and allowing the onset of transcription.

However, phosphorylation of the CTD is probably not the sole function of TFIIF, as RNA polymerases that lack the CTD, as a result of proteolysis, are active for transcription and still require TFIIF to initiate transcription *in vitro*. Because TFIIF consists of five subunits, it seems likely that this factor carries out activities other than phosphorylation of Pol II. In particular, the interaction of TFIIF with TFIIE and -J may be important for transcription, although what

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TFIIE and -J do remains undefined. Pol II that lacks a CTD can still initiate transcription *in vitro*, which may indicate that phosphorylation of the CTD is only one component of the trigger that uncouples Pol II from the preinitiation complex.

Moreover, the establishment of TFIIF as a specific CTD kinase does not rule out the possibility that other kinases may be involved in transcription initiation or even post-initiation events. For example, the cell-cycle regulating kinase, M-phase promoting factor, also phosphorylates the CTD, perhaps at different residues to those phosphorylated by TFIIF. Intriguingly, this phosphorylated form of Pol II is actually abrogated for transcription. So other kinases may target different residues of the Pol II CTD to modulate

transcription by distinct signalling mechanisms, whereas TFIIF appears to serve as part of the switch to kick RNA polymerase into the actively transcribing form of the enzyme.

The remaining five general transcription factors, many of them multiprotein complexes, possess activities that are yet to be defined. What their functions turn out to be, as we unravel the mechanisms of transcription initiation, are tales to be told another day. □

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SOLAR POWER

Towards energy storage

Prabir Dutta

EFFICIENT solar energy storage may become possible following work described by Vermeulen and Thompson on page 656 of this issue¹. The authors describe a halide-viologen complex trapped in an inorganic matrix which can store solar optical energy indefinitely.

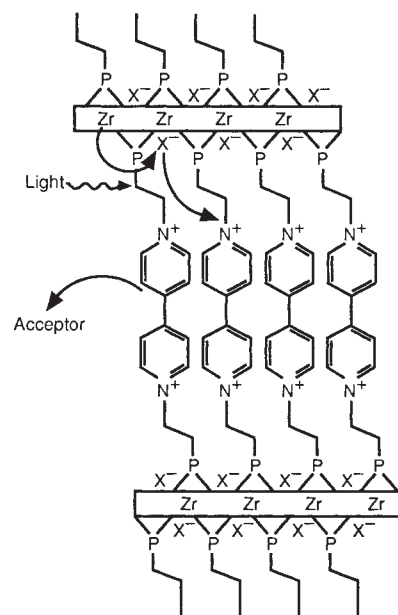
The conversion of solar energy into storable electrical or chemical energy would make an attractive alternative to the burning of fossil fuels. Photovoltaic systems currently in use are a step along this direction, and great strides are being made in improving these and other systems based on photoelectrochemistry^{2,3}. One strategy commonly adapted in work on permanent storage is to use light-induced separation of charge in electron donor-acceptor compounds. The redox products, in principle, can then be used in subsequent reactions to release the stored energy. The success of this strategy, however, depends on being able to thwart the back reaction between the redox products.

In their work, Vermeulen and Thompson use a halide-viologen unit for charge separation. The blue colour of the viologen radical formed in the photo-redox reaction persists indefinitely, even in the presence of air. Evidently, this arises from the arrangement of the complex in the interlayer space of a zirconium phosphonate matrix — in homogeneous solution, the back electron transfer is facile.

Those in the field, taking their cue from natural photosynthetic systems, have long recognized that a precise and ordered assembly of molecules is required for effective photoinduced charge separation. Various strategies are being

developed. These range from elegant syntheses of donor and acceptor molecules held at specific distances and geometries by spacer groups^{4,5}, to micro-heterogeneous systems that promote the optimal assembly of donor and acceptor species⁶. Vermeulen and Thompson's report is an example of the latter category and compares well with other matrices, such as zeolites^{7,8} and sol-gel glasses⁹. In these supports, the cages and cavities promote optimal alignment as well as mobility to ensure long-lived charge separation. In Vermeulen and Thompson's example, the alignment of the interlayer viologens promotes electron delocalization over many viologen molecules. Besides, a unique feature of their system that may be responsible for the slow back electron transfer reaction is the possibility that the framework ZrO_6 unit may be donating an electron to the halide radical. Thus, in the case of this novel support, not only are the photoactive molecules being aligned in specific geometries, but the framework itself is participating in the charge transfer process.

There are several aspects of this system that will need to be improved before it becomes a practical energy storage material. First, its photoresponse needs to be moved to longer wavelengths so that a larger fraction of the solar spectrum is absorbed. One way would be to use iodide ions, which give viologen charge-transfer bands that extend beyond 600 nm. Replacement of zirconium by other metals could promote the electron transfer to halide radicals and so increase the charge separation yield. Also, one must be able to extract the



energy stored, in which case the viologen in the interlayer must be accessible. This is a problem because of the matrix's close-packed lattice. One strategy might be to promote charge migration along the packed viologens followed by transfer of the electron from the solid to a species in solution phase whose reduction potential is lower than that of interlamellar viologen (see figure).

Although making these changes, and making them work, will be no trivial matter, the new work clearly shows that it should be possible to make solar energy storage devices and maybe batteries in the not-too-distant future. □

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Airblast scars on Venus

H. J. Melosh

ON the morning of 30 June 1908 a tremendous blast ripped through the taiga near the Tunguska river in Siberia, levelling about 2,000 km² of dense forest, snapping off metre-diameter trees like matchsticks, and charring the clothes of a trader 60 km from the centre of the explosion. In spite of the strong atmospheric shock wave, no crater was ever found. Today, we believe that the event was caused by a meteorite or comet about 100 m in diameter that entered the Earth's atmosphere at high speed, broke up under aerodynamic stresses, and deposited most of its energy before it could reach the ground.

As the atmospheric effects of meteorite entry can be so dramatic on Earth, it is not too surprising that features suggestive of airblast effects have now shown up on the Magellan images of Venus. Venus's atmosphere is about 50 times more dense than the Earth's atmosphere at the surface, and the density in a strong blast wave on Venus approaches 0.5 gm cm⁻³, so the Venusian atmosphere is capable of transmitting far more energy than the Earth's. In addition, the higher density of Venus's atmosphere means it has a greater stopping power for incoming meteorites, making it doubly potent as a medium for shock-wave energy transport.

Although the possibility of airblast effects on Venus was originally proposed on theoretical grounds¹, it was not until the Magellan radar images were analysed last year that definite features attributable to blast waves were recognized. Nearly half of all Venusian impact craters are surrounded by dark margins of low radar backscatter², whose characteristics imply that the surface is smooth on the scale of the Magellan radar wavelength (12.6 cm). Whether these margins are deposits of fine material (suggested by occasionally sharp lobate contacts with brighter surrounding plains), or were originally rough, rocky surfaces pulverized by the shock wave (suggested by the often diffuse contacts with surrounding material), or are something else entirely, is still unclear.

Some dark margins are themselves surrounded by brighter areas, indicative of greater roughness at larger ranges from the crater. In addition to dark margins that surround recognizable craters, there are also nearly 400 diffuse dark patches typically 30–50 km in diameter that lack an associated crater. It is proposed² that these patches were produced, Tunguska-like, by shock waves from meteorites too small to strike the surface directly.

The most comprehensive theory of these craterless dark patches (sometimes referred to as dark halos, dark shadows or, unofficially, splotches) published to date recently appeared in the pages of the *Journal of Geophysical Research*³. In this paper K. J. Zahnle traces the entry, deceleration and fragmentation of meteorites encountering Venus's atmosphere. Because of large aerodynamic stresses, even the strongest meteoroids break up at altitudes near 50 km. The resulting cluster of fragments spreads out as it further penetrates the atmosphere, and the larger cross-sectional area greatly increases both drag forces and atmospheric energy deposition.

For meteoroid masses of about 10¹³ kg, the energy deposition becomes catastrophically large at altitudes of 5–20 km (depending on entry angle), and the terminal phase of entry resembles a point explosion in which most of the meteoroid's original kinetic energy is transferred to shock waves in the atmosphere. Smaller meteorites stop at higher altitudes and deposit less energy, whereas much larger objects retain their original high velocity and produce craters on the surface. Zahnle finds that this atmospheric entry model adequately explains the dearth of craters smaller than about 30 km in diameter. Fragmentation is an essential aspect of this model: without it one would expect more than 43,000 craters in the size range 2–32 km on Venus, whereas in fact only about 150 are observed on the nine-tenths of the planet studied so far⁴.

Finally, Zahnle uses his model to examine the strength of the blast wave produced by meteoroids that do not quite reach the surface. Matching his results to the observed craterless dark patches, he concludes that the dark regions were subjected to blast pressures no smaller than 0.2–1 gigapascals. Although the precise mechanism by which such pressures reduce roughness is still speculative, it seems at least plausible that loose rocks lying on the surface might be shattered and pulverized by these tremendous forces (for comparison, reinforced concrete buildings are levelled by blast pressures of only 0.2 megapascals).

Several other investigators besides Zahnle are studying the effects of blast waves on the surface of Venus. In an intriguing set of experiments, Russian scientists A. A. Provalov, B. A. Ivanov and colleagues^{5,6} simulated the shock wave caused by the entry of a meteorite by detonating an explosive string over a metre-square aluminium plate coated

with loose sand. They found that the blast wave itself had no noticeable effect on the sand, but that the sand was cleared from the centre of the plate long after the blast wave's passage by a downward post-shock flow of gases following the trajectory of the simulated meteoroid. In addition, Provalov and Ivanov noted the phenomenon of 'back venting' that ejected sand from small holes drilled in the plate. As a blast wave passes over the loose, permeable soil the overpressure in the wave drives atmospheric gases into it. After the shock's passage the gases expand, carrying small soil particles into the air above the surface. This phenomenon is responsible for raising dust near terrestrial explosion tests at shock overpressures as small as 0.1 megapascals. Although it is not yet clear how this process might act on Venus, the Russian lander images of its rubbly, fractured surface suggest that back venting could occur there, and the resulting blanket of fine-grained material might contribute to the radar-dark appearance of the terrain near impacts.

Finally, T. Takata, T. J. Ahrens and R. J. Phillips⁷ have estimated fluid particle velocities behind blast waves generated by kilometre-scale meteorites traversing Venus's atmosphere, finding speeds theoretically capable of displacing rocks 10-cm across or larger more than three crater radii from an impact site. They speculate that such transient high-speed winds could grind down and transport surface debris, leading to the observed dark margins.

Although progress has clearly been made in identifying the process which may affect the surface of Venus near impacts, much more remains to be done before the case is closed on the origin of dark margins. None of these models addresses the smoothing effects of blast waves in detail, nor are the details of the atmospheric flow near an impact clearly understood, except perhaps for the case where the meteorite is small enough that it stops in the atmosphere (although even here more work on fragmentation at high velocities, where meteorite compressibility is not negligible, and on the effects of oblique entry is needed). The influence of a dense atmosphere on the course of meteorite entry, atmospheric passage and final crater formation (including ejecta deposition) is still very

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much a frontier in the study of planetary impact cratering.

We can look forward to further theoretical and experimental work on the interaction of strong blast waves with natural surfaces, perhaps supplemented with observations from old nuclear-airburst and high-explosion tests. Theoretical studies (mostly numerical hydrocode computations) of impacts on a planet with an atmosphere are needed to understand how the meteorite's blast wave, the expanding plume of ejected debris, and the ambient atmosphere interact to produce the observed deposits.

Such processes are of minimal importance on planets with very thin atmospheres, such as Mars, but are much more important on the Earth and Venus. On Earth, however, fluvial erosion has erased most ejecta deposits of large impact craters. Venus, with its dense atmosphere and low erosion rates, promises to be a marvellous natural laboratory for the study of impact-atmosphere interactions. □

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BIOSENSORS

NO release for good measure

Solomon H. Snyder

NITRIC oxide is one of the most important messenger molecules in biology, mediating the killing by macrophages of tumour cells and bacteria, dilating blood vessels in response to neurotransmitters, and serving as a neurotransmitter in the brain. One of the major limitations in understanding these diverse roles of nitric oxide (NO) has been the difficulty in measuring it, as it is a free radical with a half-life of only a few seconds. On page 676 of this issue Malinski and Taha¹ describe an unusual microsensor that can measure NO concentrations both at the external surface of single cells and in their interior, and which may eventually help provide a more detailed grasp of its physiological functions.

The first indication that nitric oxide functions as a biological mediator came from mice with a genetic deficiency of macrophages². Nitrate excretion in the urine is normally increased in inflammatory conditions, but was absent in these mice; it was subsequently found that macrophages generate NO by oxidation of arginine using a nitric oxide synthase enzyme. Removal of arginine from the incubation medium, or the use of arginine derivatives that are NO synthase inhibitors, prevents macrophages from killing tumour cells and bacteria³.

It has been found that the relaxation of blood vessels by acetylcholine is impaired when the endothelium is removed, probably because the endothelial receptors respond to acetylcholine by generating an endothelial-derived relaxing factor (EDRF) which diffuses to the muscle layer to provoke relaxation⁴. The amount of NO released in this process is sufficient to account for the EDRF activity⁵.

In the brain the NO synthase enzyme is localized to discrete populations of neurons that are selectively resistant to neurotoxic damage in Huntington's dis-

ease and stroke⁶, and in the peripheral autonomic nervous system it is found in populations of neurons that regulate peristalsis in the intestine, in the nerves to the erectile tissue of the penis, and in neurons to the posterior pituitary and to the adrenal medulla. In these systems NO acts as a neurotransmitter: the synthase enzyme in the nerves causes NO release upon nerve stimulation. Inhibitors of NO synthase therefore block physiological responses. Of the three forms of NO synthase that have so far been cloned, the neuronal⁷ and endothelial^{8,9} enzymes bind calmodulin and are activated by calcium, but the macrophage enzyme¹⁰⁻¹² is activated by synthesis of new enzyme in response to immune stimulation and is insensitive to calcium. Nitric oxide synthase is one of the most sensitive enzymes in biology, in keeping with its responsibility for fine-tuning the levels of a major physiological mediator. The development of a sensor that can rapidly respond to minute changes in concentration of NO *in situ* is therefore a considerable technical advance towards monitoring the release and distribution of NO in the body.

In designing their microsensor, Malinski and Taha have taken advantage of the ease of oxidation of NO and its ready diffusibility¹. It was already known that highly conducting polymeric metalloporphyrins can catalyse the electrochemical oxidation of small molecules. The authors created a polymer of porphyrin with nickel as the central metal. As the polymer also reacts with NO-related anions such as nitrites, they coated the polymer with Nafion, a substance that is permeable to NO but blocks the diffusion of nitrite.

The resultant electrochemical detector is highly sensitive and specific. It detects a concentration of NO of about 10 nM in solution. In a volume equivalent to a

single cell, it can readily detect 10⁻²⁰ mol of NO, which is substantially less than the amount of NO produced in individual cells. The authors applied their method to measuring NO in blood vessels and, in the process, established definitively that NO is endothelial-derived relaxing factor. This factor is released by endothelial cells in response to molecules that dilate blood vessels, such as acetylcholine and bradykinin, and diffuses from the endothelial cells to the adjacent smooth muscle cells, causing them to relax so that the blood vessel dilates⁴. When the microsensor is placed on the surface of endothelial cells in the aorta, it detects substantial levels of NO released into the medium after the administration of bradykinin. Implantation of the microsensor into a nearby smooth muscle cell reveals that NO concentrations have increased in response to bradykinin.

The NO microsensor will help to probe several unresolved features of NO function. When activated, both macrophages and neutrophils generate NO (ref. 3), which before it kills tumour cells and bacteria presumably hits lymphocytes and other cells in the vicinity of the inflammatory response. Does NO affect these other cells? The sensor should be able to detect NO in any such cellular targets. Nitric oxide synthase occurs in selected neuronal populations in which NO seems to act as a neurotransmitter⁶; the sensor may reveal whether firing of these neurons increases NO only in adjacent neurons or whether glia and other cells are also affected. It may be that NO is a participant in models of synaptic plasticity, such as long-term potentiation and long-term depression, because inhibitors of NO synthase interfere with these processes⁶. Intracellular monitoring of NO concentrations could also clarify the sources and targets of NO in these instances. □

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Spectral breaks for theorists

Matthew G. Baring

MUCH has been made over the past nine months of the spatial distribution of astronomical γ -ray bursts, as revealed¹ by the BATSE (Burst and Transient Source Experiment) detector aboard the Compton Gamma-Ray Observatory satellite, in the hope this will tell us something of their origin. But more information may be forthcoming from the detailed spectra of individual bursts, recorded by the same apparatus. Schaefer *et al.*² report finding distinct breaks at energies between 400 keV and 2 MeV in the spectra of five out of 18 bright bursts recorded by BATSE, which may be related to the source conditions.

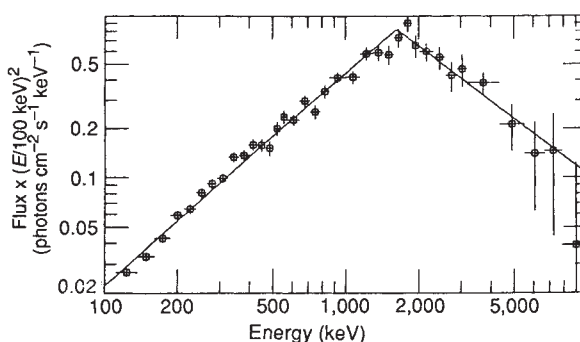
The principal finding from BATSE is that the bursts are distributed isotropically over the sky, but that there is a comparative deficit of distant sources. The common inference is that the bursts are cosmological in origin³, and cannot be explained in terms of the conventional hypothesis⁴ involving neutron stars in our own (disk-like) Galaxy. The neutron-star hypothesis was most attractive because of the appearance of absorption features at less than 50 keV in burst spectra recorded by the early KONUS experiments. These were naturally explained as cyclotron absorption lines in 10^{12} -gauss magnetic fields expected of neutron stars. In 1988, the Ginga X-ray satellite observed sources with double absorption dips, equally spaced in energy^{5,6}. This dramatically bolstered the cyclotron-line interpretation, and the idea that γ -ray bursts originate near neutron stars became the vogue until the BATSE detections of the past year.

High quality

The high quality of the BATSE burst data has made it possible now to discriminate between theoretical models of their spectra. The correct prediction of the shape of the energy spectrum near the break can now be made a test for any model, and should be a focus of future theoretical investigations. All reasonable source models for bursts require that an enormous luminosity is generated in a relatively small volume. Only a few radiation processes can accomplish this while also providing relatively sharp breaks in the γ -ray continuum.

Two-photon production of electron-positron pairs ($\gamma\gamma \rightarrow e^+e^-$) is the most familiar radiation mechanism for attenuating γ -ray spectra of astrophysical

sources. For a power-law spectrum of negative logarithmic slope (spectral index), the probability of attenuation increases with energy so that above some γ -ray threshold energy there is an absorption turnover and break in the spectrum. The higher the photon density in the source, which means a greater distance to the source for a given detected flux of radiation, the lower is the energy at which the break occurs. This property was used to place burst sources that showed no evidence of attenuation below about 3 MeV (like many of the Solar Maximum Mission and BATSE



Best break yet: BATSE spectra reveal onset of γ -ray-attenuating mechanisms. (Event 910814-69275, from ref. 2.)

sources) within 1 kiloparsec of the Earth⁷ (much closer, say, than the Galactic Centre). The breaks generated by $\gamma\gamma \rightarrow e^+e^-$ are sharp enough to account for the results presented by Schaefer *et al.*² but may not produce the appropriate slopes for the spectra above the breaks.

For extremely high source luminosities, as required for extragalactic bursts, the pairs produced will give a dense gas that will scatter the photons through the Compton process and produce a thermal peak in the spectrum, contrary to the data. To avoid this, the pair production rate must then be dramatically lower. This can be achieved if the γ -rays emerge in a beam more tightly collimated than known in extragalactic jets. The absence or presence of a spectral break should severely constrain the degree of radiation beaming in any model with a cosmological population of burst sources.

In the strong magnetic fields surrounding neutron stars, there is also the exotic quantum process of spontaneous production of an electron-positron pair by a single photon, $\gamma \rightarrow e^+e^-$. This process has a threshold around 1 MeV, and will efficiently attenuate a γ -ray burst spectrum above this. It is a popular mechanism for the production of pairs in

theories of pulsar magnetospheres. Averaging over suitable radiation angular distributions, $\gamma \rightarrow e^+e^-$ usually causes a sharp spectral break⁸ between 1 and 3 MeV, compatible with two of the spectral breaks displayed by Schaefer *et al.*². But in such strong fields, synchrotron radiation is likely to be the dominant emission process, yielding relatively steep spectra so that only one of the BATSE examples could fit a model with $\gamma \rightarrow e^+e^-$ attenuation.

Relativistic electrons

A third mechanism for generating spectral breaks is the inverse Compton scattering of X-ray photons (perhaps from an accretion disk or a stellar surface) by relativistic electrons, which give up some of their energy to the radiation. This could apply to bursts in both galactic and cosmological environments. In low magnetic fields, the continuum photons may be scattered many times to generate a peak around 1 MeV of quasi-thermal shape⁹.

But nothing like this is seen. In the magnetic fields of neutron stars, inverse Compton scattering is a process that is resonant at the cyclotron energy (about 30 keV) and can generate spectral breaks at any energy above this¹⁰. Despite its flexibility, this mechanism is quite constrained in its range of spectral slopes at

energies below the break, and is probably compatible with only two of the broken-power-law BATSE observations presented by Schaefer *et al.*²

It is unlikely that the BATSE spectra will be surpassed this decade, and astrophysicists will be challenged to make the most of them. Certainly, no definitive answers are forthcoming yet. It is a pity that the most distant bursts will be too faint to yield spectra, as any redshift in their features relative to those in brighter bursts would confirm a cosmological origin. But fitting the energy of any break and the spectral slopes around it is still a powerful tool for unravelling the mechanism of γ -ray bursts. □

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Fishing in Src-infested waters

Gregory A. Petsko

MACROMOLECULAR structures are always beautiful and usually stimulating, but structural information is not always what is most needed at a particular time. One field that is ripe for more of such data is signal transduction. That need is met, in part at least, by the high-resolution crystal structure of the SH2 domain of the Src tyrosine kinase complexed with a phosphotyrosine-containing peptide, reported on page 646 of this issue by Waksman *et al.*¹. Together with the structures of two other SH2 domains obtained by NMR in the absence of bound ligands^{2,3}, it should have a considerable impact on many areas of cell biology.

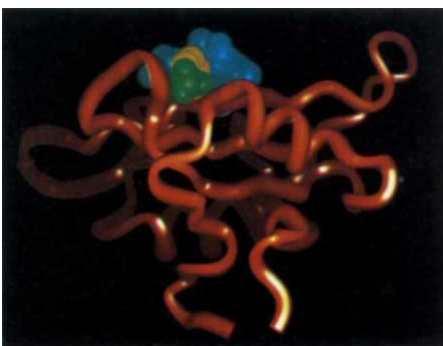
When growth factors bind to the extracellular domains of their receptors, they can induce receptor dimerization⁴, which brings the cytoplasmic tyrosine kinase domains of individual monomers into close proximity. In the dimer, the kinase domains cross-phosphorylate one another at several intracellular tyrosine residues, thereby transducing the signal of growth factor binding to the interior of the cell. Further passage of the signal requires that the phosphotyrosine-containing peptides on the kinase be recognized by other proteins.

A specific recognition domain, identified in the proto-oncogene tyrosine kinases Fps and Src and designated SH2 (for *src* homology domain 2), is responsible for recognition and binding in a large number of proteins⁵⁻⁷. SH2 domains are about 100 amino acids long and can be located in any region of a protein sequence⁵. They are compact, self-contained modules; isolated SH2 domains bind specifically and tightly to phosphotyrosine-containing peptides^{8,9}. The proteins into which they are inserted may be tyrosine kinases themselves, or soluble proteins. Often, SH2 domains are accompanied by smaller, 45-residue SH3 (*src* homology domain 3) domains, but each of them can be found alone. The function of the SH3 domain is less clear. A plethora of SH2 and SH3 domains have now been found in proteins involved in signal transduction, from phospholipase C γ , phosphatidylinositol-3-OH kinase, *ras* p21 GTPase-activating protein, to protein tyrosine phosphatase IC, the putative transcription factor *Vav* and in other proteins of unknown function⁵.

Why so many different but related SH2 domains in the Src-infested waters of the cytoplasm? When combined with multiple phosphorylation sites on the growth factor receptor kinase domain, each with its own neighbouring se-

quence, this arrangement allows a single growth factor receptor to turn on a specific subset of 'downstream' cellular enzymes. Each kinase may recruit slightly different SH2 domain-containing proteins, based on differences in affinity for specific phosphotyrosine-containing peptides.

Sequence comparisons among SH2 domains, which in fact share only 25-40% identity, suggest that they all have a common fold that is unrelated to other



Structure of the modular SH2 domain of the Src tyrosine kinase bound to a tyrosine-phosphorylated peptide. Red ribbon represents the polypeptide backbone, with amino and carboxy termini close together (bottom). Bound peptide is shown by spheres representing each atom (tyrosine ring, yellow, remainder of the peptide, blue). The phosphate group (green) is specifically recognized by a deep pocket in the SH2 domain.

proteins. The structures of the isolated form of the SH2 domain of the Abl tyrosine kinase² and the amino-terminal isolated SH2 domain of phosphatidylinositol-3-OH kinase, determined by NMR, indicate that the SH2 fold is indeed novel. It is a central, three-stranded, antiparallel β -sheet with a smaller antiparallel sheet at one end. Single amphipathic α -helices pack on either face of this sheet. The domain is hemispherical in shape, with the residues implicated by mutagenesis experiments in peptide binding located on the flat surface.

The two NMR structures are very similar but also seem to have some differences, suggesting that the sequence differences between SH2 domains may modulate the packing of secondary structural elements, at least in the absence of ligand. The plasticity of structure between different SH2 domains may be important for conferring sequence-specific peptide binding on individual SH2 domains. Strikingly, both groups observe that the short loop connecting the first and second β -strands in the sequence is ill-determined, and they

speculate that it may be flexible and fold down over the recognition site when phosphorylated peptides bind.

The most detailed and interesting results come from the crystal structure of the SH2 domain of Src itself complexed with phosphorylated peptides¹. The overall fold of the protein is identical to that observed for the Abl and phosphatidylinositol-3-OH kinase SH2 domains, despite the limited sequence similarity. Reasonable models for nearly all SH2 domains can probably be built on the basis of these three structures using the positions of known invariant residues⁷. Waksman *et al.* note that the structure is ideally modular (see figure), allowing this domain to be inserted anywhere in a protein sequence without disturbing its fold or function. In contrast to the deep grooves used to bind peptides in proteases, antibody-combining sites and the proteins of the major histocompatibility complex, the Src peptide-binding site is a fairly flat surface perpendicular to the β -sheet. The phosphotyrosine recognition site, however, is a deep pocket in this surface.

Some of the differences between this structure of an SH2-peptide complex and those of the isolated SH2 domains are clearly correlated with peptide binding. Three positively charged residues (Arg 155, Arg 175 and Lys 203 in Src) interact directly with the bound phosphotyrosine, His 201 indirectly. These residues are rigidly held near one another by the phosphotyrosine, as are the amino-terminal α -helix and the so-called 'phosphate-binding loop', which is identical to the loop between the first and second β -strands referred to above (although Waksman *et al.* number their β -sheet strands differently). The phosphate-binding loop is folded down over the phosphotyrosine and several residues in the loop make hydrogen bonds to phosphate oxygens. In the structure of the uncomplexed Abl SH2 domain, the amino-terminal helix is rotated relative to its position in the Src structure, opening up the peptide-binding site and moving apart the two arginines that correspond to Arg 155 and Arg 175 in Src.

The interaction between SH2 and phosphotyrosine thus seems to be an example of induced fit, necessitated by unfavourable electrostatic interactions between positively charged side chains in the uncomplexed protein that are overcome when the negatively charged phosphate group binds.

Recognition of the phosphotyrosine is accomplished by a surprising set of interactions. To begin with, the site is not particularly hydrophobic, and no non-polar residues pack around the tyrosine ring. Three positively charged residues

participate, but only one (Arg 175) makes exclusive contact with the phosphate group. Its terminal guanidinium nitrogens each donate a hydrogen bond to a phosphotyrosine oxygen, a classic mode of binding between arginine and phosphate. Lysine 203 forms a platform for the aromatic ring, with its positively charged amino group making an enthalpically favourable, weakly polar interaction¹⁰ with the partial negative charges on the pi cloud of the ring. This same type of interaction is used by one of the guanidinium nitrogens of Arg 155 on the opposite face of the tyrosine ring, while its imino nitrogen makes a conventional hydrogen bond to a phosphate oxygen. Dual recognition of the aromatic group and the phosphate group of phosphotyrosine by the same side chain provides a specific binding site for the phosphorylated form of the amino acid; it is too elegant for it not to be used in other situations.

Individual SH2 domains recognize different peptide sequences containing phosphotyrosine^{8,9}. The two different crystal structures solved by Waksman *et al.* provide reassurance that the binding of phosphotyrosine itself is likely to be the same in each case and the more variable and flexible regions of the SH2 domain may dictate specificity. Such a mechanism is implicated in the binding of the growth hormone receptor to alternative peptides in the hormone¹¹. The open-faced nature of the peptide-binding site implies that SH2 makes a sandwich with the phosphotyrosine-containing protein to which it binds. The phosphate group thus functions as a covalent form of what Ringe has called a 'schatchen' (from the Yiddish for marriage broker)¹², a matchmaker that brings together two macromolecules by interacting simultaneously with them both. The union between SH2 domains and tyrosine-phosphorylated residues then nucleates the assembly of the multiprotein complexes responsible for triggering cell growth and differentiation. □

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Knocking out memory's door

Daniel V. Madison

LONG-term potentiation (LTP) of synaptic transmission has long been touted as a neural mechanism for memory, but that relationship has been supported by little experimental evidence. Now, two papers in *Science*^{1,2} greatly strengthen the link between LTP and memory by showing that a gene that controls LTP also controls the acquisition of a certain form of memory in mammals. In these studies, mutant mice lacking the gene for the α -isoform of the calcium-calmodulin-dependent multifunctional protein kinase II (α -CaMKII)³, were generated by gene targeting. Mutant animals were shown to have deficits in acquiring a spatial learning task, and LTP was greatly impaired in hippocampal slices taken from them.

The link between LTP and memory remained purely hypothetical for many years, and was based largely on the realization that LTP, the persistent increase in synaptic efficacy that results from brief high-frequency activation of excitatory synapses, had properties that seemed to be similar to some properties of memory. Recently, experimental findings began to correlate LTP directly with a particular form of memory for spatial relationships. Animals given pharmacological antagonists to the NMDA subtype of glutamate receptor were unable to carry out these^{4,5} or other⁶ learning tasks, and also had blocked LTP^{4,5}. The deletion of the CaMKII gene produces results that are not subject to the same doubts raised by the use of NMDA antagonists, in that the gene deletion does not appear to affect normal synaptic transmission. Still, because of the possibility that such deletion may also impair other functions in the hippocampus, or in other parts of the brain, the link between LTP and memory remains mostly correlative. Nonetheless that correlation now takes on added force.

The studies also provide helpful information on the mechanistic role that α -CaMKII plays in LTP. Studies using pharmacological or pseudosubstrate kinase inhibitors suggested that CaMKII was necessary for LTP^{7,8}, but the lack of selectivity of the probes used made it impossible to draw conclusions about the isoforms of the enzyme responsible. Deletion of the α -CaMKII not only indicates that this particular molecule is important in LTP, but also implies that it plays a regulatory rather than an obligatory role. This implication arises because a small percentage of hippocampal slices from mutant animals were able to generate LTP that was essentially indistinguishable from that seen in tissues

taken from wild-type animals. An oddity of this result was that LTP, when it appeared, was of about the same magnitude as seen in wild-type tissues, even in field potential recordings which detect transmission at many synapses simultaneously. In most of the slices from mutant animals, LTP was completely absent. That LTP in mutant tissues may be expressed in an all-or-none fashion means that the LTP deficit caused by the removal of a regulatory function of CaMKII may be overcome in many synapses simultaneously in a given tissue. This conclusion is not easily explained under current understanding of LTP mechanisms.

One of the most remarkable aspects of the new work, both in terms of memory and LTP, is just how selective the effects of deleting the α -CaMKII gene are in the mutant mice. Such selectivity is especially notable given that α -CaMKII makes up a large percentage of the total protein in the brain, and a particularly large portion of protein in synaptic specializations⁹. Perhaps because of the late developmental expression of α -CaMKII, the anatomical organization of the neocortex and hippocampus seem to be unaltered, and synaptic function (save LTP) and the behaviour of the animals (save the deficit in spatial memory) are very similar to wild type.

There are, of course, issues of specificity that will need to be resolved. CaMKII is present in other parts of the brain besides the hippocampus, and could exert effects on memory by processes unrelated to LTP. Closer examination of these animals may reveal some further differences from wild type, and it will be necessary to rule out the possibilities that the additional deficit in paired pulse synaptic facilitation does not contribute to the memory deficit, or that the animals' hyperexcitability does not unduly influence the behavioural testing. One particularly promising approach to deal with these questions may lie in the development and use of specific targeted mutations in brain-

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expressed genes, or in the use of region-specific promoters to target transgenes to desired areas of the brain.

Probably the outstanding aspect of this work is that it demonstrates that the techniques of gene targeting can be used successfully to study the processes underlying memory, and perhaps other behaviours, in mammals. In another study, preliminary results of which have been announced at meetings, targeted gene knockout has been employed to probe the relationship between LTP and memory. Seth Grant, Tom O'Dell, Paul Stein and colleagues, in the laboratories of Eric Kandel and Philippe Soriano, have demonstrated that independent mutations in the *fyn* gene, but not three other tyrosine kinases (*src*, *yes* and *abl*), produce a dramatic increase in the

threshold for inducing LTP, and a corresponding blunting in the memory for spatial tasks. The *fyn* mutation is also associated with developmental abnormalities of the hippocampus.

All in all, we have a promising beginning in the use of gene targeting to study mammalian learning and memory. Not only does gene targeting improve on the traditional pharmacological approach, but it also opens up the possibility of using designed mutants to study the biochemical consequences of deleting selected enzymes. □

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genes is initiated during early development in the most anterior region of the embryo and follows a temporal sequence, beginning with *Otx2* at 7.5 days post coitum and progressing through *Otx1*, *Emx2* and *Emx1* (*Emx1* being first detectable at 9.5 days post coitum). The spatial distributions of all four transcripts share an important feature with those of Hox genes (see figure): as with the latter, *Otx* and *Emx* transcripts are distributed in overlapping regions of the developing neural tube, giving the potential for their protein products to be employed in either a combinatorial or a hierarchical manner to specify cell fate. Because the sites of expression are more rostral than any Hox gene, do the *Otx* and *Emx* genes constitute part of a (thus far elusive) set of genes involved in controlling diversification within the vertebrate head?

Although this hypothesis is appealing, two differences from the Hox genes must be mentioned. First, the four *Otx* and *Emx* genes show a nested series of posterior expression boundaries, in contrast to clearly nested anterior expression boundaries of the Hox genes. Second, the order of succession of Hox gene expression boundaries parallels the chromosomal order of these genes within a gene cluster¹; there is, however, no

DEVELOPMENT AND EVOLUTION

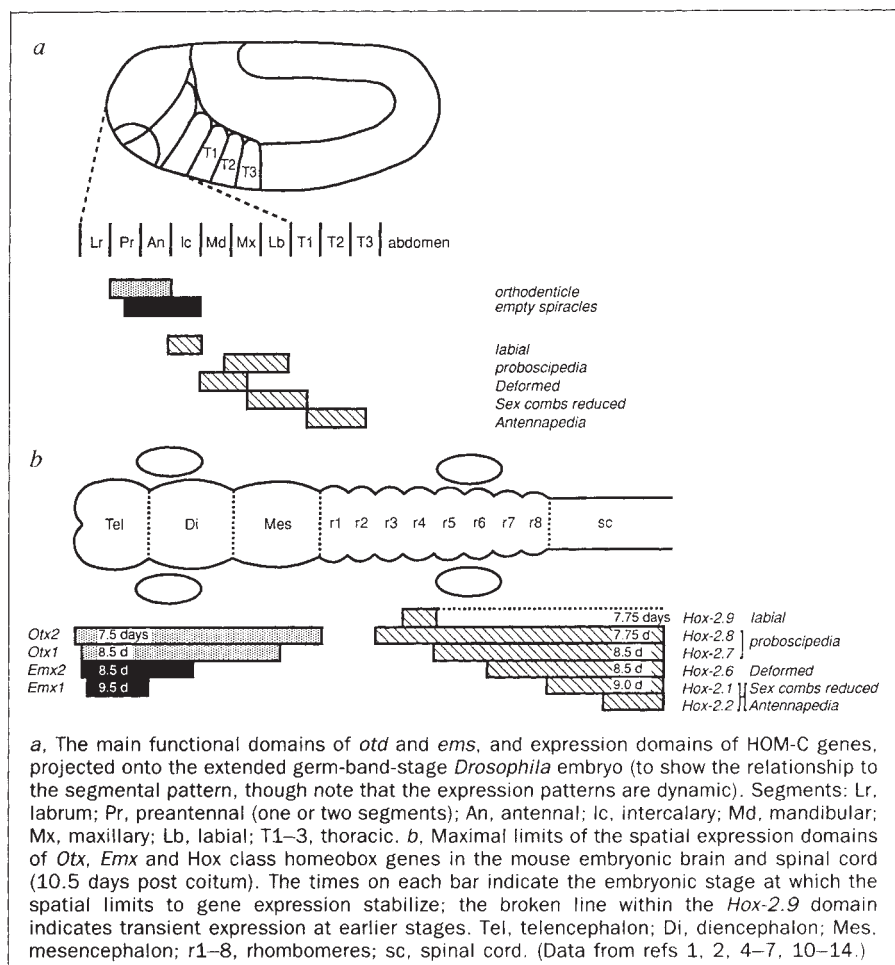
Mice and flies head to head

Peter Holland, Philip Ingham and Stefan Krauss

SEQUENCE homologies between *Drosophila* and vertebrate genes are now commonplace, but striking examples of parallels in their deployment and function, such as those exhibited by the vertebrate Hox and invertebrate HOM clusters¹, remain the exception. Certainly, few might have predicted that the obscurities of insect head segmentation could teach us much about the development of the vertebrate brain. Yet, on page 687 of this issue², Simeone *et al.* describe a remarkable similarity in spatial expression patterns between the *Drosophila* head 'gap' genes, *orthodenticle* (*otd*) and *empty spiracles* (*ems*), and their mouse homologues.

Both *otd* and *ems* have only recently been identified as gap genes that play a role in head segmentation in *Drosophila*³⁻⁶. Both encode homeodomain proteins and are expressed in overlapping domains at the anterior pole of the blastoderm stage embryo (see figure), patterns of expression that depend on the activity of the anterior morphogen Bicoid⁴⁻⁶. From mutational studies it seems that *otd* and *ems* are involved in the establishment of different head segments⁴⁻⁶. Given that no HOM-C genes are known to be required for the differentiation of these segments, it has been proposed that both genes also have a homeotic function, specifying the identity of the segments whose boundaries they define. This second function is difficult to prove, because the relevant segments are eliminated from embryos mutant for either gene; nonetheless, such a notion provides an intriguing clue in thinking about what their vertebrate counterparts do.

Two cognates of each gene (designated *Otx1* and 2, and *Emx1* and 2) have been cloned from mice by virtue of high sequence conservation between their homeoboxes^{2,7}. Expression of these



evidence for clustering of *Otx* and *Emx* genes. Indeed, clustering seems unlikely because *Otx* and *Emx* class homeoboxes are quite divergent in sequence, arguing against recent common ancestry by tandem gene duplication.

Viewed from an evolutionary perspective, what is to be made of the clear spatial expression difference between Hox genes (in the hindbrain and spinal cord) and *Otx* or *Emx* genes (in the forebrain and midbrain)? If roles for Hox, *Otx* and *Emx* genes in body regionalization evolved early in metazoan radiation, the fundamental molecular dichotomy within the vertebrate neural tube is a legacy from events preceding the evolution of the vertebrate head; nonetheless, there are likely to be adaptive consequences. If the different homeobox gene families are under different modes of regulation (for example if the tight clustering of Hox genes restricts mutational change) then subsequent variation and adaptive radiation will have been constrained to different extents anterior and posterior of the midbrain/hindbrain boundary. We suggest this could be a molecular basis for the comparative evolutionary plasticity of the vertebrate forebrain and midbrain, but conservation of hindbrain morphology, during vertebrate evolution. This hypothesis predicts that *Otx* and *Emx* gene expression will show greater interspecies variation than will Hox gene expression.

In mammals, the expression domains of the *Otx*, *Emx* and Hox genes encompass less than the total rostrocaudal axis of the neural tube, and even in regions of expression there seem to be too few genes to allow fine-grained coding of position. Consequently, if these genes control regionalization of the embryonic brain, they probably do so in combination with other anteriorly expressed regulatory genes. Some candidates for such genes have already been isolated, for example members of the *Pax*, *POU*, *Dlx*, *En*, *Krox* and *Wnt* gene families. Another gene worth searching for would be a vertebrate homologue of *buttonhead*, a *Drosophila* gene that forms part of a trio of head-specific gap genes, along with *otd* and *ems*³.

Might additional genes, responsible for the development of other body regions, have conserved functions at the whole organism level? The *Cdx/cad* and *Evx/eve* class homeobox genes are good candidates: *Drosophila cad* and vertebrate *Cdx* genes share one expression site, the embryonic gut⁸, whereas vertebrate *Evx-1* and the locust *eve* gene (though not its *Drosophila* counterpart) are expressed in posterior mesoderm⁹.

Struck by the comparable sites of expression of *Otx/otd* and *Emx/ems* class genes in *Drosophila* and mammals, we

have argued in favour of evolutionary conservation of function. But similarity in expression need not be a reflection of identical function; experimental manipulation of expression of *Otx/otd* and *Emx/ems* genes in vertebrates and *Drosophila* will be needed to test this idea. Even if similarity in function were to be demonstrated experimentally, it would not be unequivocal evidence for evolutionary conservation because similar roles could have been adopted independently in the two lineages. An important approach to resolving this dilemma will be the comparative analysis of these genes in a wide range of metazoan taxa.

The gross similarity between the *Otx/otd* and *Emx/ems* expression patterns in mammals and flies may be striking, but there are a few differences that could point to convergent evolution. For example, is it valid to compare the simple epithelium of the *Drosophila* blastoderm, where the *otd* and *ems* genes are first expressed, with the neural tube of mammals (the site of *Otx* and *Emx* expression)? Account has to be taken of several *Drosophila* genes that are involved in blastoderm patterning and that may have been secondarily co-opted from older functions in patterning the nervous system (*eve* and *ftz*, for example)^{9,10}. Whether such co-option to roles in the blastoderm occurred for *otd* and *ems* is not known, though it is worth noting that both genes are also expressed in neuronal cells^{5,6}.

Until more comparative analyses and experimental manipulations are performed, it is impossible to resolve these issues. The deployment of the *Otx/otd* and *Emx/ems* genes in mammals and flies may be the result of convergent evolution — but we suspect they will turn out to represent a surprising case of evolutionary conservation. □

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Billing Big Brother

WHILE wrestling with DREADCO's intractable tax returns recently, Daedalus began to ponder the thermodynamic truth that information always has a cost. So the government, in demanding a mass of information for free, was in fact robbing him. Bureaucratic states rob their citizens and businesses of an endless stream of costly data. Some of them let the citizens fight back with equally costly 'freedom of information' demands. But Daedalus feels that all information should be traded at a fair price. This is particularly true of personal information. We instinctively resent its being demanded, and still more its being stolen without our knowledge.

So DREADCO technologists propose to graft a billing system onto the computer network that increasingly handles bureaucratic data. Any search will then trigger an appropriate payment. Every time somebody adds your name and address to a junk mail list, you will be told, and paid. Every time some bureaucrat accesses your social security number, you'll get paid. Every time your bank gives the revenue authorities or a credit agency details of your account, you'll get paid. Every time some legal outfit or regulatory body demands a ridiculous set of statistics from your company, it will have to pay for them. The prices of the various types of data will be set by classic market forces. A few active 'market makers' will bargain hard over their data, and these negotiations will fix the going rates for the mass of uncontested transactions.

Bureaucracy will thus be tempered by market realities. Once they have to pay a fair price for it, government agencies will think twice about demanding information. Ludicrously intrusive taxes such as VAT, which gives the government a cut of, and therefore a need to know about, every single transaction in the whole country, will suddenly become less attractive. Policemen and social security snoopers will be more selective with their suspicions — particularly as some shrewd citizens will specialize in appearing suspicious, so as to profit from the resulting enquiries.

Better than any data-protection law, the free data-market will tell us who holds what information about us, and will pay us for providing it. But Daedalus fears that many agencies will cheat, and bypass his billing system. If the police tapped your phone, for example, they would probably not want to admit it by paying you for the data thus acquired. But should the case come to court, they would have to explain the source of their evidence. A shame-faced police payment might slightly sweeten the pill of conviction.

David Jones

Hydrothermal supermounds

SIR — High-resolution sidescan sonar images have revealed huge mounds (up to 190 m tall) on the Reykjanes Ridge (northern Mid-Atlantic Ridge) southwest of Iceland. They occur on the eastern flank of a neovolcanic zone, lying along the plate boundary close to 58° N (arrowed in Fig. 1). Similar, albeit smaller, steep-sided features, found elsewhere along the Mid-Atlantic Ridge, have a hydrothermal origin.

The mounds, which cast long acoustic shadows towards the southeast (arrowed in Fig. 2) and away from the sidescan sonar vehicle, are located in the north-west corner of the image. The vehicle's

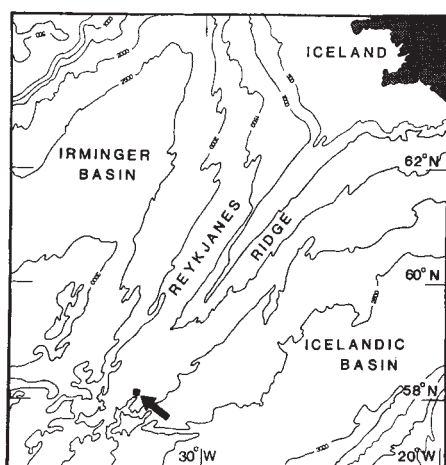


FIG. 1 Bathymetric map of the Reykjanes Ridge locating the site of the supermounds (arrowed).

track is northeast–southwest, and passes across the far top left-hand corner of the image. The size and shape of the mounds can be estimated from the length and shape of their acoustic shadows. We know from multi-beam echosounder data that the sea floor beneath

the shadows is virtually flat (Parson *et al.*, personal communication), and that the vehicle passed the mounds at an altitude of 400 m, at a range of 500 m. We have identified four mounds, which are aligned northeast–southwest, and appear bell-shaped from their insonified sides. In order of position from northeast to southwest, the heights of the mounds above the sea floor (in metres) are: 190, 180, 135 and 185 (± 10). Their basal widths, measured parallel to the vehicle track, are: 150, 90, 135 and 160 (± 6); and their maximum slope angles (in degrees) are 57, 68, 71 and 54 (± 6), respectively.

Elsewhere on the image the sea floor is characterized by volcanic ridges and faults trending northeast–southwest and parallel to the plate boundary. A conical volcano in the lower centre of the image, and a prominent shield volcano in the bottom left-hand corner (arrowed in Fig. 2), allow a comparison with the mounds. These unique images were obtained using the new towed ocean bottom instrument from the Institute of Oceanographic Sciences, Deacon Laboratory (IOSDL), which has a resolution of about 2 m (ref. 1).

The origin of the mounds remains enigmatic. They may be individual volcanoes, but their extremely steep slopes make such an explanation unlikely. One of the steepest flanked submarine volcanic seamounts reported to date, on the Mid-Atlantic Ridge at 24° N (Lawson *et al.*, personal communication), has a slope angle of about 30°. The mounds imaged on the Reykjanes Ridge are more than 20° steeper than this, so are unlikely to be volcanically constructed.

Alternatively, the mounds may have a hydrothermal origin. This explanation is

consistent with their bell-like shapes and neovolcanic setting. We know from active hydrothermal fields on both the East Pacific Rise and the Mid-Atlantic Ridge that steep mounds and chimney-like structures are common. These arise from the precipitation of metal-sulphides around escaping high-temperature fluids. Hydrothermal mounds at the 'Snake-pit' field (23° N on the Mid-Atlantic Ridge) are beehive-shaped, 40–100 m long by 20 m wide and up to 50 m high, with flank angles of between 50° and 70° (ref. 2). Similarly, the 'TAG' hydrothermal mound (26° N of the Mid-Atlantic Ridge) is 50–100 m high and approximately 200 m across³. Have we seen a set of hydrothermal supermounds on the Reykjanes Ridge? Their shape suggests that we have, but their size is greater than any hydrothermal mounds or chimney-like structures reported previously.

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CH⁺ in the Red Rectangle

SIR — The CH⁺ molecule was first discovered in diffuse interstellar clouds more than 50 years ago¹. Although a great deal of research has been undertaken, the mechanism of its formation and the reason for its high abundance are not well understood². In a recent spectroscopic study³ of the star at the centre of the unusual Red Rectangle biconical nebula, the discovery of a set of emission lines near 4,200 Å was reported but the carrier was not identified. By comparing the reported wavelengths with known laboratory spectra, we show here that these lines arise from CH⁺. Detection of the CH⁺ molecule in a nebula presents a new opportunity to investigate the reason why CH⁺ is such an abundant astro-physical molecule. The CH⁺ spectrum will also provide a very useful probe of the chemical and physical conditions in the Red Rectangle.

The published Red Rectangle emission spectrum³ covers the range 4,215–4,245 Å; the wavelengths of the six reported lines are given in the table, where comparison is made with the laboratory data⁴ for CH⁺. The excellent agreement between the observed and laboratory wavelengths is conclusive evidence that CH⁺ is the carrier of the emission lines.

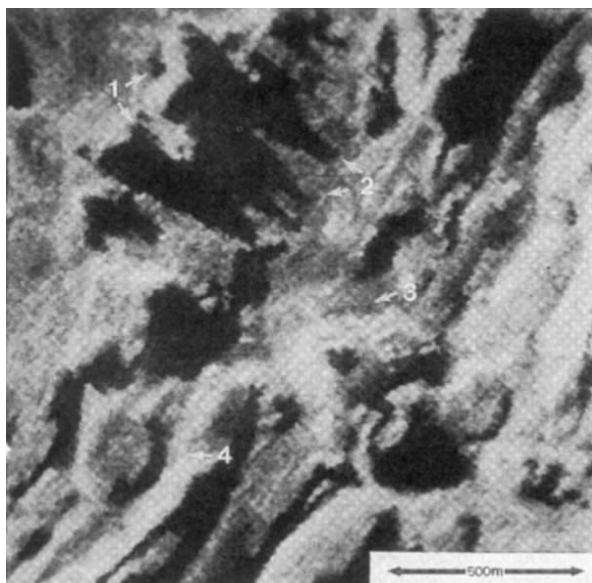


FIG. 2 High-resolution sidescan sonar image, produced using the IOSDL new towed ocean bottom instrument of the supermound site on the Reykjanes Ridge (Mid-Atlantic Ridge southwest of Iceland). The image, 1,500 × 1,500 m, was insonified from the top left-hand corner. It is similar to a monochrome photograph, with strong acoustic returns as bright tones and weak ones as dark tones. Arrowed and numbered on the image are: (1) the supermound; (2) shadows cast behind the supermounds; (3) a conical volcano; and (4) a shield volcano.

COMPARISON OF RED RECTANGLE EMISSION LINES WITH LABORATORY WAVELENGTHS FOR CH⁺

Observed wavelength (Å)	Laboratory wavelength (Å)	Assignment	Difference (Å)
4,225.82	4,225.70	R(3)	0.12
4,227.14	4,227.06	R(2)	0.08
4,229.45	4,229.35	R(1)	0.10
4,232.70	4,232.55	R(0)	0.15
4,237.67	4,237.56	Q(1)	0.11
4,239.49	4,239.38	Q(2)	0.11

Observed data from ref. 3; laboratory data from ref. 4.

In studies of CH⁺ in diffuse interstellar clouds, the R(0) absorption line at 4,232.55 Å alone is detected because the temperature is sufficiently low for only the lowest rotational energy level to be populated. The observation of six lines of CH⁺ from the Red Rectangle shows that the molecule is warmer than in diffuse clouds.

Using published quantum mechanical line strengths⁵ we have determined the rotational temperature of CH⁺ to be 120 ± 50 K, using a least-squares fitting routine. The observed (calculated) intensities of the lines relative to the Q(1) line are: Q(1) 1.0 (1.0), Q(2) 0.9 (1.0), R(0) 0.6 (0.7), R(1) 0.6 (0.6) and R(2) 0.5 (0.4). The value of about 120 K is very low compared with the temperature of the star at the centre of the Red Rectangle nebula; moreover, the CH⁺ line-widths of about 0.25 Å are two to three times narrower than those of the stellar absorption lines of atomic carbon. These data confirm that the molecule is in the surrounding nebula rather than the star.

Although CH⁺ has been known in interstellar clouds for many years, it appears that the Red Rectangle is the only nebula in which CH⁺ has so far

been detected. The identification of CH⁺ in the Red Rectangle, coupled with the recent recognition of some of the unassigned 'diffuse interstellar bands' in emission from the same nebula^{6,7}, leads to the interesting question of whether the high abundance of CH⁺ in diffuse clouds might be related to the presence of diffuse band carriers in these clouds.

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limb perfusion of human patients shows that not only sarcomas, but also melanomas and squamous cell carcinomas⁹, respond to the antitumour activity of TNF (plus interferons). In an isolated limb perfusion trial involving 52 melanoma patients, 90% complete remission and 10% partial remission were obtained. The antitumour effect involves activation of polymorphonuclear leukocytes and of the endothelial cell bed, with over-expression of adhesion molecules, which is much stronger in the tumour vasculature than in the normal one. This results in coagulative and haemorrhagic necrosis exclusively limited to the tumour tissue.

Although it is correct that a better patient selection might be important in improving the results obtained with TNF-related treatment, we nevertheless believe that such a selection should not be based primarily on the tumour histology, but rather on (as yet insufficiently identifiable) parameters predicting the susceptibility towards the systemic toxicity of TNF, properties of the tumour and its microvascular system, and perhaps on previous treatment. Extensive research on the mechanism of action of TNF (alone or combined with interferons) at the cellular level has provided no evidence for a correlation of the susceptibility with the embryological origin of the cell, but rather with the degree of malignancy¹⁰.

In our view, it would be unfortunate to deny this treatment to those melanoma, sarcoma and carcinoma patients who do qualify for treatment; protocols and conditions need to be further explored.

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Coley's vaccine and TNF therapy

SIR — C. O. Starnes in his Commentary¹ drew attention to the remarkable success that Coley achieved around the turn of this century in the treatment of cancer patients with his 'mixed toxins'. On the basis of an analysis of the historical records, Starnes proposed that bacterial toxin-related and tumour necrosis factor (TNF)-related treatments should be restricted to those patients suffering from sarcoma or lymphoma, and that further efforts should be concentrated on a search for so far unidentified factors made in response to Coley's vaccine. We agree with him and with Rook² that TNF does not account for all the effects observed after administration of Coley's toxins, but we nevertheless also believe that Starnes' conclusions may in some respects have been misleading.

First, despite the relatively disappointing results obtained during earlier clinical trials in which TNF was used as an

ordinary chemotherapeutic agent, the often dramatic beneficial results obtained in recent clinical trials with high-dose TNF in isolated limb perfusion³, in the treatment of ascites⁴, or other forms of loco-regional treatment⁵, leave little room for doubt about the clinical relevance of TNF (preferably in conjunction with other cytokines) as an anticancer treatment.

It is to be hoped that we will learn how to control the shock-inducing effect in a selective way so that these results may be extended to treatments involving the administration of TNF in the general circulation.

Second, in addition to the animal studies showing beneficial effects in sarcoma models, other animal studies in which TNF was used in conjunction with interferons showed a similar beneficial effect for melanomas^{6,7} and for carcinomas⁸. More important, isolated

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Moving with the times

J. R. S. Fincham

The Dynamic Genome: Barbara McClintock's Ideas in the Century of Genetics. Edited by Nina Fedoroff and David Botstein. Cold Spring Harbor Laboratory Press: 1991. Pp. 422. \$55.

BARBARA McClintock has earned fame in three different ways. In the 1930s her brilliant and incisive cytogenetic studies were of great importance in the consolidation of the chromosome theory of heredity, and were recognized as such at the time. From the mid-1940s, she applied the same qualities of acute observation and clear thinking to an extraordinarily detailed, though entirely pre-molecular, analysis of movable genetic elements in maize. This apparent undermining of the accepted stability of the genome was difficult for most geneticists to come to terms with. It was only after movable genetic elements (prophages, transposons) had been characterized at the DNA level in bacteria, and the molecular analysis extended from bacteria back to maize, that McClintock's pioneering observations were assimilated into the mainstream. The third aspect of McClintock is her unique standing as an individual. Her solitary style of work, total independence of thought and extraordinary record of getting things right have together elevated her to the status of a prophet in the eyes of some.

The volume under review, produced in celebration of McClintock's ninetieth birthday, deals with all three aspects of her career. Fedoroff and Botstein have assembled a collection of essays by scientists who have interacted with or been influenced by McClintock, and have interspersed them with reprints of some of her own most seminal papers (a minor complaint is that the sources are unidentified except in so far as they appear on the reproduced pages). The essays are a mixed bag, ranging in merit, I would say, from a masterly review by Marcus Rhoades of the history of maize genetics and McClintock's part in it, to G. Albrecht-Buehler's three pages of laboured irony directed against unidentified or imaginary denigrators. The other contributions are mostly personal accounts of how various pieces of work in areas related to McClintock's interests came to be done.

In some cases the authors acknowledge initial inspiration or personal advice from McClintock; in other cases the connection was made only *post hoc*. All are worth reading as records of successful science as it should be conducted. There is nothing about squabbles over priority, fussing about intellectual property or anxieties over the next grant

appreciated genetic mechanisms, both of radical importance. The range of mutations shown by her mutable alleles is now known to be due mainly to excisions, frequently imprecise, of the element that had been inserted at the gene locus. McClintock herself, at least initially, was inclined to put her mutable alleles into the same category as *Drosophila* position-effect variegation, with proximity of transposable elements in the maize systems having the same kind of effect on gene expression as the proximity of rearranged heterochromatin in the fly. She clearly envisaged self-perpetuating changes in chromatin structure of the kind now called epigenetic. This idea was largely pushed into the background by the more recent molecular demonstrations of element excision

as the prime source of mutability. But the epigenetic changes of potency often shown by McClintock's elements, and her even more challenging observations of "presetting" of gene activity (40 years ahead of 'imprinting' in the mouse genome), are something else, representative of a class of phenomena of great and growing current interest.

James Shapiro, Gerald Fink and Bruce Alberts have the most to say in this book about McClintock's biological philosophy. Shapiro believes that her supposed "mysticism" is really just the courage to admit lack of understanding — combined, however, with a determination to find out. Fink recalls her reiterated challenge to him in conversation: "You think you understand it but you don't." Does this just mean that things are more complicated than we yet know? This is a truism; after the glorious and genuinely simplifying discoveries of molecular biology, we are now stuck, for the indefinite future it seems, with the prospect of complexity leading to yet more complexity. What McClintock means, I think, is that although we are sorting out the molecular mechanisms, we have still not grasped their significance for the whole organism.

She has always regarded her transposable elements as functions belonging to the organism and declines to join the growing consensus that they are just relatively benign molecular parasites. In originally naming them "controlling" elements, she meant that they not only controlled aberrant gene activity but were likely to be agents of normal control as well. It has to be said that this idea has gathered no support

IMAGE
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REASONS

Barbara McClintock (1947): an extraordinary record of getting things right.

application. One expects accentuation of the positive in a volume like this but, even so, one cannot help feeling that those were happier times.

Nina Fedoroff wraps up the volume with an expert account of where our knowledge of maize transposable elements now stands. Intensive molecular investigation in numerous different laboratories has now clarified many questions of detailed mechanism. However, the armies of advanced technology are, even now, only just coming to grips with the range of phenomena identified by McClintock using only her own hands and eyes.

As we can now see, McClintock's observations and inferences pointed not just to one but to two previously un-

over the years. We now know a great deal about normal regulatory mechanisms in the genomes of higher eukaryotes and, so far, transposable elements figure only as occasional interfering interlopers.

In more recent years, and especially in her Nobel lecture, McClintock has placed more emphasis on transposable elements as one way, among others, of restructuring the genome in response to stress. There is evidence that the maize elements are sometimes activated by chromosome breakage induced by other means. They can then promote further breakage and segmental rearrangement. Does this make them a part of the problem or a part of the solution, or both? Is the restructuring just random

disruption, from which natural selection can, given rare good luck, fashion a new order? Or does the organism, as sometimes seems to be implied, have foresight, conjuring up just the kind of restructuring that the occasion demands? That would indeed seem to verge on mysticism.

I, for one, would celebrate Barbara McClintock not as a prophet but just as a supremely perceptive and individual scientist. If she has a message, it may be that we should always believe our observations, however bizarre they may seem. They may be trying to tell us something. □

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Ivan Roitt's introductory book *Essential Immunology* (Blackwell, 7th edn, 1991), can be annoying in an advanced text, even though here they are well used and plentiful. Despite the extra detail, the experimental basis of the findings is often not delved into. Rather, each chapter is a synthesis of 'current' work typical of well-edited reviews, with ample reference to original articles and helpful tables, including one of CD nomenclature.

Writing an advanced immunology text is certainly a daunting task, because the more detail one goes into the more likely it is that the book will be out of date before the ink is even dry on the pages, as occurs here in some sections on tolerance. Overall, though, Male and his co-authors have succeeded in providing a fine supplementary text for an advanced immunology course. □

Defending the immune system

David W. Scott

Immunology. By Janis Kuby. *W. H. Freeman*: 1992. Pp. 585. \$46.95, £19.95. **Advanced Immunology, Second Edition.** By David Male, Brian Champion, Anne Cooke and Michael Owen. *Gower Medical*: 1991. Pp. 304. \$69.95, £39.50 (hbk); \$45, £24.95 (pbk). (Distributed by Raven Press in the United States.)

AN immunology sage once remarked that you can't understand any immunology until you know all of immunology. As a teacher, I have uttered another version of the same maxim: "Don't worry, this will all fit together later." Part of this dilemma is reflected in the organization of most immunology courses and texts. Where should they begin? Some authors or teachers describe first the cells and organs of the immune system, whereas others introduce the uninitiated to the chemistry of the molecules (the nature of antigens, immunoglobulin structure and genetics) and to their interactions. Even the major histocompatibility complex, no longer relegated to the topic of transplantation, can be an orphan belonging to neither of these categories or to both. Typically, both of these books have different organizational styles aimed at overlapping audiences. Nonetheless, both avoid the ultimate dilemma of teaching immunology by providing an 'overview' of the immune system, taking the form of an introduction in Kuby's *Immunology* and a review in Male *et al.*'s *Advanced Immunology*.

Immunology is aimed at senior undergraduates and introductory graduate students. It abounds with experimental and historical examples, as well as clear illustrations. Each chapter has a good set of problems and answers to help students review important issues. Kuby

writes with warmth and a love of the field, placing emphasis on the main immunological principles and the molecular biology underlying current understanding. In the second chapter, for example, she discusses molecular techniques that provide a basis for understanding immunoglobulin rearrangements and the structure of T-cell receptors.

There are several minor errors, however, which may be bothersome to some teachers. Some of these are errors of omission, whereas others reflect the delay between writing and publication. For example, the importance of CD4 helper cells in cytotoxic T-cell responses is overemphasized — recent studies, such as those with mice harbouring knock-out mutations, demonstrate that helper cells are not needed for most cytotoxic responses. And several of the study questions emphasize minor points at the expense of more important ones. Nevertheless, the overall structure and approach of the book should make it a very useful and excellent introduction that will stand up strongly in comparison with other good texts, such as E. S. Golub and D. R. Green's *Immunology: A Synthesis* (Sinauer/Freeman, 2nd edn, 1991) and A. K. Abbas *et al.*'s *Cellular and Molecular Immunology* (Saunders, 1991), the last of which is aimed primarily at medical students. Although Kuby's text also includes several chapters on clinical immunology, it is geared more towards those students who enjoy learning about the experimental basis and the history of the field.

Advanced Immunology is less broad, focusing instead on a smaller part of the field. It is intended to be an advanced treatise, devoting more time and detail to each subject. There is particular emphasis on lymphocyte activation and immune regulation. The clear illustrations, which are influenced by those in

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New in paperback

- **Living Fossil: The Story of the Coelacanth** by Keith S. Thomson. Norton, \$9.95. For a review see *Nature* **353**, 29 (1991).
- **Food of the Gods: The Search for the Original Tree of Knowledge** by Terrance McKenna, a "radical history of plants, drugs and human evolution". Rider, £9.99. For a review see *Nature* **356**, 635 (1991).
- **Language and Species** by Derek Bickerton, a detailed account of the origins and evolution of language. University of Chicago Press, \$15.95, £11.25. Reviewed in *Nature* **348**, 372 (1990).
- **Morphogenesis: The Cellular and Molecular Processes of Developmental Anatomy** by Jonathan Bard. Cambridge University Press, £17.95, \$37.95.
- **The Concise Oxford Dictionary of Zoology** edited by Michael Allaby. Oxford University Press, £7.99. In his review in *Nature* (**354**, 325; 1991), Ralph A. Lewin described the dictionary as "a fine compendium of unquestionable use".
- **Engines of Creation: The Coming Era of Nanotechnology** by Eric K. Drexler. Oxford University Press, £7.99.
- **Changing Order: Replication and Induction in Scientific Practice** by H. M. Collins, now with a new afterword, is published by University of Chicago Press, \$14.95, £10.25.
- **Exons, Introns and Talking Genes: The Science Behind the Human Genome Project** by Christopher Wills. Oxford University Press, £8.99. Reviewed by J. S. Jones in *Nature* **354**, 323 (1991).

An ideal Universe?

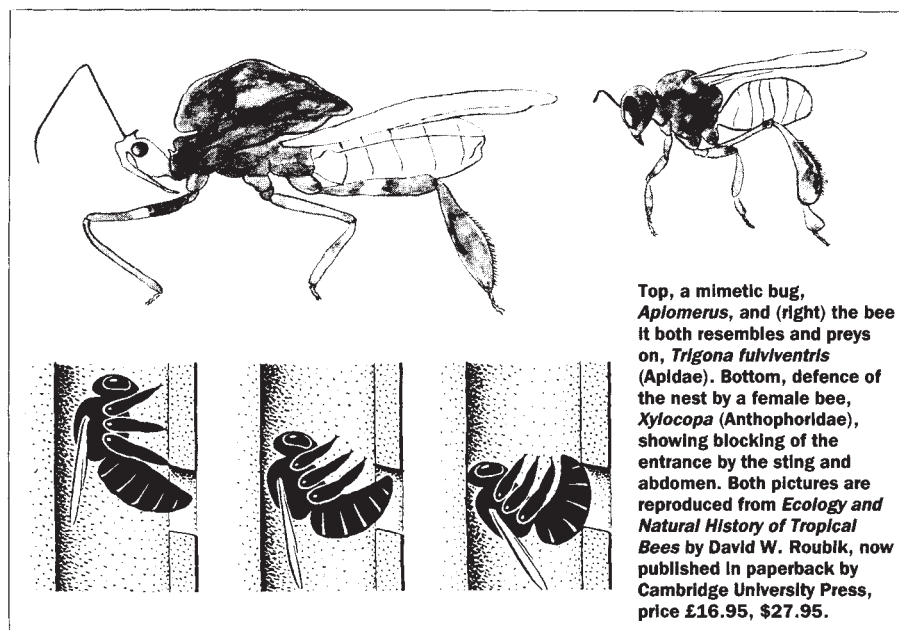
A. Rupert Hall

Descartes' Metaphysical Physics. By Daniel Garber. *University of Chicago Press*: 1992. Pp. 389. \$60, £47.95 (hbk); \$23.95, £19.95 (pbk).

BY giving a mighty kick to a stone, that British arch-empiricist Samuel Johnson proved to his own satisfaction the reality of matter. The pain in his foot caused by striking a large mass assured the sensational existence of solid bodies. By contrast, the refounder of idealism in Europe, René Descartes, went to great intellectual lengths to prove by reasoning alone that the stone we seem to see or feel is not an illusion; to Descartes, Johnson's sore toe demonstrated only his mental awareness of the stone, not its real existence. In Descartes' view, our sense of something is no demonstration of its reality, for bodies perceived by our senses and bodies truly existing are not the same thing — as any physical scientist will agree. Galileo made the same distinction more simply by his famous separation of the primary from the secondary qualities of bodies, a distinction echoed by Boyle and Locke.

To a modern scientist, as indeed to Laplace, it may seem strange that Descartes deduced the reality of 'bodies' (the Universe) from a logical demonstration of the existence of an active God, rather than vice versa (which was the way of the natural theologians Henry More, John Ray and Isaac Newton). In Cartesian metaphysics, the existence and rational structure of the Universe were warranted by our subjective knowledge of its Creator, who cannot deceive. Descartes does not seem to deal with the position that, regardless of whether the Universe is real or a dream, the Universe must be of equal interest to us, because we are conscious of inhabiting it and being profoundly affected by it. Having proved to his own satisfaction that 'bodies' are real, Descartes accepted the consequence that the human mind can form a true image of them, although this must be very different from our first naïve impressions of the composition of things.

A most important contribution by Descartes to the development of modern physics was his rejection of the 'qualities' of things as real and basic; he preferred instead to reduce them to matter and the size, shape and motions of its constituent particles. As Daniel Garber rightly points out, Descartes was indebted for this notion to the Greek atomist tradition, which was revived in the sixteenth



Top, a mimetic bug, *Aplomerus*, and (right) the bee it both resembles and preys on, *Trigona fulviventris* (Apidae). Bottom, defence of the nest by a female bee, *Xylocopa* (Anthophoridae), showing blocking of the entrance by the sting and abdomen. Both pictures are reproduced from *Ecology and Natural History of Tropical Bees* by David W. Roubik, now published in paperback by Cambridge University Press, price £16.95, \$27.95.

century and expounded as a valid philosophy of nature by Pierre Gassendi. Descartes' corpuscular philosophy was the more successful, not only because it was carefully purged of anti-Christian overtones and was more detailed and imaginative in its kinematics, but also because it was creatively linked by Descartes to the mathematical treatment of physical puzzles such as the refraction of light, and the rainbow. In turn, Descartes' system was the antecedent for later mechanist philosophies such as those of Huygens and Leibniz, while Newton (during his creative middle life, at any rate) preferred the atoms-and-the-void concept which he took (ultimately) from Gassendi, enriching and transforming this by his own mechanism of forces.

After the establishment of the reality of matter, the next most important issue for Descartes was the inevitability of motion, for the movement of material particles was, in his view as in that of the atomists, the origin of all phenomena. Motion came originally only from God, and therefore could be neither lessened nor enlarged in the duration of the Creation. It could only be transferred from particle to particle, from gross body to gross body, and solely by the impact of one upon the other. Here, at the very heart of his system, Descartes encountered great difficulties that he failed to overcome — although he himself admitted no such failure. For example, in dealing with the motion of light, he treated it now as a particle travelling from a source, at other times as a pressure in a particulate medium. This, and other curious procedures, was justified in Descartes' mind by the contention that a potential motion can be equated to an actual movement. (Thus a particle under pressure in some direction

will move as soon as its motion is unobstructed.) Descartes' ideas led him to formulate an incorrect — indeed, almost absurd — theory of collisions, soon revealed as such by others. Garber is perhaps too kind to Cartesian mechanics, and to Descartes' confidence that the rival, geometrical ideas of movement stemming from Galileo were useless.

For all that, Garber's analysis of Descartes' *a priori* account of the Universe is acute, learned and detailed. Descartes' intellectual debt to Isaac Beeckman of Dordrecht from the discussions the two men had in 1618 is fully acknowledged. Garber's analysis is not always easy to follow: the subject is difficult and technical. Moreover, the book combines an unfolding of Descartes' mature system (found chiefly in the Latin *Principles of Philosophy*, 1644) with an investigation of its evolution from the draft *Le Monde* (early 1630s); the reader has therefore to follow a critical investigation of Descartes' thought about bodies and motion while also taking account of the changes in his thought. That said, the book is very well argued and is founded upon a complete familiarity with Descartes' writings. As the use of the adjective 'metaphysical' in the title indicates, this is wholly a philosophical discussion, which does not extend to consideration of the connections between Descartes' reconstruction of the bases of thinking about nature, and the two wonderful optical treatises of 1637 (*Dioptrique* and *Météores*) — or indeed to the transition in the *Principles* itself from these foundations to Descartes' specific, hypothetical corpuscular mechanisms for the explanation of phenomena. □

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Growing pains

P. E. Bryant

Terman's Kids: The Groundbreaking Study of How the Gifted Grow Up. By Joel N. Shurkin. Little, Brown: 1992. Pp. 317. \$22.95.

DURING the first ten years of this century, Alfred Binet, a distinguished French psychologist, devised the first successful intelligence test, which immediately became one of psychology's most formidable achievements and also one of its greatest headaches. The intelligence test, without doubt, is the most effective and widely used instrument invented by psychologists, but it lacks a theoretical base. We know that it works quite well: it will, for example, predict fairly accurately how well children will do at school, in the immediate future at any rate. But we do not know even now what intelligence tests measure, and we are equally hard put to say with any coherence what intelligence is. The contrast between the test's practical success and its theoretical ambiguity is extremely stark.

A great deal of the test's immediate practical success was due to the work of Lewis Terman, an American psychologist who produced the first transatlantic version of Binet's test — the Stanford Binet — and then went on to devise other tests of his own. He had no doubt about the importance of his work. "There is nothing", he argued "about an individual as important as his IQ except possibly his morals." Naturally, Terman wanted to establish the value of what he was doing, and in 1921 he began a research project whose main aim was to show that people's lives are deeply affected by their intelligence levels as measured by his and by other intelligence tests. He and his colleagues set out to identify all the children in the Californian school system whose IQ scores were in the top one per cent of the population. Having selected 1,470 such children, the researchers then gathered a large amount of detailed information about their families and homes, their history, their health and their performance at school.

But the main feature of the study was that it was longitudinal. Terman wanted to find out what would happen in adulthood to these able children, whom he insisted, rather generously as it turned out, on calling "geniuses". He kept in touch with his group of "Termites", as they were soon called, and continued to gather information about them, both by correspondence and by systematic research until his death nearly 40 years later. By and large, he showed that

these children grew up to be successful adults, or at any rate more successful than the average person, and this result has been taken by many as establishing the validity of intelligence tests.

Since Terman's death, other psychologists have continued to collect data on these remorselessly studied people, who by now are in their 80s and 90s. The sheer amount of information on Terman's children is both daunting and impressive, but it does not compensate for several basic flaws in the design of the project. The most serious of these was the omission of a control group. Most of the Termites came, not surprisingly, from prosperous and well educated families. So it is quite possible that their better than average progress through life was determined by their family backgrounds and not by their IQ. Terman should have compared his geniuses with a group of children who came from much the same backgrounds but did not have as high IQs. Without this comparison we cannot be sure that the study has told us anything about the value of IQ *per se*.

Joel Shurkin's book is partly a popular account of the project and partly a biographical picture of Terman himself. The author was given access to the detailed records on some of the people in the study, and he has obviously wrestled with the voluminous reports that have been written about the project. His book is at its strongest when it deals with the journalistic fringes of the topic. He has some quite interesting things to say, for example, about the wide fame achieved by the project, which has often been featured in newspapers and in popular journals. He also writes entertainingly about the effects of being a Termite. It seems that many of them came to rely heavily on Terman's advice and support (Terman got one of them into Stanford University, for example), to the extent that Terman's influence on the lives of his subjects may have had a considerable effect on his results.

The book is weak, though, on the conceptual and scientific issues. Although Shurkin briefly mentions the lack of a control group, he is never clear about the controls that are needed. Nor does he write well about the purpose of the project, which was to show the power and the value of intelligence tests. The book is particularly weak when he deals with quantitative evidence. In discussing the particular difficulties encoun-



Charles Cagniard de la Tour's siren (1819). This diagram is reproduced from *The Science of Musical Sound* by John R. Pierce, the revised edition of which is now published in paperback by Freeman, price £17.95, \$19.95. (For a review of the first edition see *Nature* 308, 791; 1984.)

tered by the women in the project, Shurkin says, "One would have to factor in sexual discrimination in the working world to understand these figures in their context" — a suggestion that is neither practicable nor even comprehensible. Shurkin is sometimes plainly illogical about the data. Terman, in one of his later reports, had divided the Termites into three subgroups: the A's who had done particularly well in life, the C's who had had the least success, and the B's who were somewhere in between. This division prompts one of Shurkin's oddest statements: "The proportion of Jews in the A group was double the proportion of Jews who were C's despite the fact that only 10 per cent of the Terman kids [that is, the total sample] were Jewish." This howler looks particularly odd in a book about highly intelligent people.

Shurkin is also prone to making rather wild and unsubstantiated claims, such as: "He [Terman] eventually had to confront the connubiality of madness and genius." If Shurkin means by this that Terman or anyone else has shown a connection between superior talents and instability he is wrong, and in fact Terman made the opposite claim.

A great deal has already been written about Terman's fascinating, but sadly flawed, study. This new book is not a valuable addition to the genre. □

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Formation of harzburgite by pervasive melt/rock reaction in the upper mantle

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Many mantle peridotite samples are too rich in SiO₂ (in the form of orthopyroxene) and have ratios of light to heavy rare earth elements that are too high to be consistent with an origin as the residuum of partial melting of the primitive mantle. Trace element studies of melt/rock reaction zones in the Trinity peridotite provide evidence for reaction of the mantle lithosphere with ascending melts, which dissolved calcium-pyroxene and precipitated orthopyroxene as magma mass decreased. This process can account for the observed major and trace element compositions of lithospheric mantle samples, and may accordingly be prevalent in the upper mantle.

PERIDOTITES with calcium-pyroxene contents lower than in the primitive mantle have commonly been interpreted as the residue of large degrees of partial melting of lherzolite¹. Here we review more than a thousand bulk compositions of upper-mantle peridotite samples, and more than a hundred bulk compositions of experimental liquids reported to be in equilibrium with mantle lherzolite. We find that many mantle samples are too rich in orthopyroxene (SiO₂-rich), and have too high a ratio of light to heavy rare earth elements to be residues of partial melting from primitive mantle compositions. The review substantiates observations that abyssal peridotites (mantle samples dredged from mid-ocean ridges and associated fracture zones) have major and trace element compositions compatible with a residual origin²⁻⁴, that ophiolite upper mantle is poorer in Ca-pyroxene than is abyssal peridotite^{5,6} and that garnet lherzolite xenoliths from the continental lithosphere are generally poorer in Ca-pyroxene and richer in orthopyroxene than other mantle samples⁷.

Major and trace element characteristics of mantle xenoliths have commonly been ascribed to two separate events: early partial melting ('depleting' the major elements) and later metasomatism ('enriching' the trace elements). Here, we show that some of the major element compositions cannot be the result of melting, and most of the major and trace element compositions can be explained by a single interaction process between magma and mantle, with or without previous partial melting of the peridotite reactant. We infer that much of the mantle beneath continents and subduction-related magmatic arcs may have been modified by reactions in which Ca-pyroxene (cpx) is dissolved and orthopyroxene (opx) and olivine (ol) are produced, with decreasing magma mass. Reaction between ascending basaltic melt and mantle lithosphere will produce peridotite enriched in opx and with a higher ratio of light to heavy rare earth elements (REE), as observed in the mantle section of some ophiolites, in lherzolite massifs and in mantle peridotite xenoliths. Liquids modified by cpx dissolution and opx precipitation may later cool and crystallize cpx and opx, with or without ol, or mix with unmodified liquids and crystallize cpx with or without ol, producing a variety of pyroxene-rich lithologies.

Our hypothesis is based on a trace element study of melt-rock reaction zones in the Trinity peridotite of northern California. Reaction zones around dunite, pyroxenite and gabbroic dykes include concentric domains of dunite (>90% ol), harzburgite (>40% ol, >5% opx, <5% cpx), lherzolite (>40% ol, >5% opx, >5% cpx) and plagioclase lherzolite (>5% plag) grading outwards into unaltered plagioclase lherzolite^{8,9}. These were formed by interaction between infiltrating basaltic melt and upper mantle peridotite⁸⁻¹⁴. New ion-probe data on the trace element

composition of minerals in reaction zones show that incompatible element concentrations in the liquid increased as the reaction progressed. In addition, titanium is fractionated from the REE by this process, so that the Ti/REE ratio decreased with reaction progress. Modelling of these data shows that they are consistent with a reaction in which cpx is dissolved and a slightly larger mass of opx and ol is precipitated.

Harzburgite and dunite have often been referred to as 'depleted' peridotites, as they were inferred to have lost a melt fraction. In the reaction process described here, the solid products cannot be viewed as depleted. They are enriched in opx and/or ol, and their total mass is greater than the mass of the solid reactants. Furthermore, in many such reactions the liquid and crystal products are enriched in incompatible trace elements relative to liquid and crystalline reactants. This is in contrast to partial melting during which the incompatible element abundances in solid and liquid products decrease. It is probable that some mantle harzburgites and dunites really are depleted; they have formed as the result of large degrees of partial melting from a lherzolite parent. It is also likely, however, that many mantle harzburgites and dunites have been formed by a process of magma-mantle interaction.

Reaction zones in the Trinity peridotite

The Trinity peridotite is a large body of mantle peridotite in the Klamath mountains of Northern California which contains numerous melt/rock reaction zones^{8,9}. The reaction zones do not displace older pyroxenite bands: although the reaction zones cut across the bands, the pyroxenites are continuous along strike on either side. Dunite and harzburgite locally include trains of chromian spinel where Cr-rich pyroxenes were dissolved; the spinel trains are continuous with pyroxenite on either side of the dunite. These relations indicate that dunite and harzburgite are replacive, formed by reactions involving little change in the solid volume. Similar reaction zones occur in many ophiolite peridotites¹⁰⁻¹⁴. They cannot have formed by partial melting in a closed system, because they are as narrow as 10 cm, and are hosted by plagioclase (plag) lherzolite. The temperature difference required to form a dunite residue by partial melting of plag lherzolite is too large to be produced or sustained over 10 cm in a system closed to advective heat transfer. Therefore, it is inferred that such reaction zones form by interaction between solid peridotite and silicate melt infiltrating along grain boundaries⁸⁻¹⁴.

Reaction zones are typically symmetrical about a plane. Locally, this plane is marked by a dyke composed of dunite, pyroxenite or gabbroite inferred to have crystallized after production of the surrounding reaction zone. Nonetheless, such

dykes may indicate the nature of the liquid reactant that produced replacive dunite and harzburgite. In the reaction zones adjacent to the medial dyke, there is a typical sequence from dunite at the centre, through harzburgite, to lherzolite and finally outward to plagioclase lherzolite. Dunite may be locally absent. The zoning sequence may be developed over tens of centimetres to several metres in outcrop. The presence of even larger reaction zones is suggested by map-scale contact relationships between dunite, harzburgite and lherzolite^{8,9}.

A systematic electron probe study of these reaction zones^{8,9,15} showed that the major element composition of minerals is nearly constant although mineral proportions vary greatly. Near a large dunite, the average magnesium number $Mg\#$ ($Mg/(Mg+Fe)$) of each of the ferromagnesian silicates shows a slight increase with increasing distance from the centre of a reaction zone, from 0.89 to 0.91 (sample series 9W), whereas in a reaction zone around a clinopyroxene-rich dyke the opposite trend is observed (sample series 8W40). By contrast, we show that trace element concentrations in clinopyroxene have large, systematic variations as a function of distance in these reaction zones. As discussed by Kelemen and co-workers¹⁴⁻¹⁸, nearly constant $Mg\#$ combined with large variations in incompatible element abundance is a likely consequence of reaction between mantle-derived melts and mantle wall rock.

We analysed cpx and opx in polished thin sections of samples from three reaction zones in the Trinity peridotite on the ion microprobe, using techniques developed by Shimizu and co-workers^{19,20}. Figure 1 presents the REE data for cpx as a function of distance perpendicular to the medial plane. Some cpx crystals may be left from plagioclase lherzolite before reaction, and some of these may have partially re-equilibrated with migrating melt, whereas other cpx grains may have formed directly by crystallization from liquid during cooling. Varying trace element abundance in cpx within a single thin section may thus reflect a variety of origins, wherein crystals formed and interacted with liquid at different times in an evolving open system. The kinetics of such interactions are beyond our scope here. Instead, we discuss systematic variations between samples, and explain these with simple models which assume instantaneous local equilibrium.

To interpret our data we need to know the direction of melt flow, and thus of reaction progress, in the reaction zones. The main components of velocity were probably in the plane of the medial dykes. But geochemistry and field relations demand a component of velocity perpendicular to this plane, out of the centre and towards the periphery of each reaction zone. The geochemical evidence is that concentrations of REE and Ti in cpx systematically increase outwards from the centre of reaction zones. Despite analysis of sample suites extending more than 3 m, we have yet to find the outer limit of a geochemical reaction

zone. REE concentration in cpx in the outer samples is higher than in cpx in plagioclase lherzolite far from reaction zones. We have determined cpx composition in three such plagioclase lherzolite samples, and obtained consistent results for all samples (Fig. 1). Our data are in agreement with five neutron activation analyses of cpx separates from Trinity plagioclase lherzolite²¹, and consistent with limited whole-rock analyses of Trinity peridotite²². We infer that cpx in the reaction zones was modified by interaction with infiltrating liquid moving from the centre towards the periphery. Incompatible trace element concentration in derivative liquids increased with increasing ratio of solid to liquid, away from the centre of each reaction zone, so that samples near the periphery have higher REE and Ti in cpx than the 'far-field' plagioclase lherzolite.

Geological as well as geochemical arguments favour a component of melt migration out of the dunite zones and into the lherzolite. Contacts between dunite and harzburgite reaction zones are very sharp, of the order of one grain diameter. Dunites (with virtually no opx) abruptly change to harzburgites with >15% opx and <2% cpx. This abrupt transition is clearly visible in glacially polished outcrops wherever reaction zones have been observed. It is inconsistent with mechanisms such as advective heating of surrounding lherzolite by hot melt or advective fluxing of the lherzolite by components from migrating melt. Such mechanisms would produce gradational variation in modal opx as a function of distance. Instead, the observation of abrupt dunite-harzburgite contacts is consistent with phase saturation in a cooling, migrating magma, in which once the liquid is saturated in opx, it produces it in fixed proportions relative to ol and other solid products.

We illustrate the variation of Ti/REE with REE concentration in cpx (Fig. 1, left) by considering Yb (heavy) and Nd (light), because many geochemical processes fractionate heavy from light REE, and these elements are easily analysed in peridotite cpx. Individual reaction zones, and the data set as a whole, show a systematic decrease in Ti/REE with increasing REE concentration. Kelemen *et al.*²³ summarized data on distribution coefficients between crystal and liquid (D) for ol, opx, cpx, spinel and basaltic liquid. Whereas ol, opx and spinel (as well as plagioclase²⁴) have higher $D(Ti)$ than $D(Sm, Eu, Tb \text{ and } Dy)$, in cpx $D(Ti)$ is less than or equal to $D(Sm, Eu, Tb \text{ and } Dy)$. If the reaction zones had been produced by melting and melt extraction, with increased melt removed near the centre, then variation in Ti/REE would have been small until cpx was absent in the residua. Figure 2 shows substantial variation of Ti/REE within lherzolites. This variation, combined with the geological observations, suggests a process involving dissolution of cpx and precipitation of one or all of ol, opx, spinel and plagioclase. Plagioclase is absent in most of the reaction zones (and unstable

FIG. 1 Neodymium concentration (Nd(N)), normalized to concentration in C1 chondrites⁴⁶, in cpx from melt/rock reaction zones in the Trinity peridotite, as a function of distance. Sample series 8W40 is one side of a reaction zone around a pyroxenite dyke (pyroxenite-harzburgite contact is at 0 and the first appearance of plagioclase, plagioclase-in, at about 30 cm). Sample series TP90-9 is one side of a reaction zone around a tabular dunite body. (Dunite-harzburgite contact at 0; plagioclase-in at about 80 cm). Sample series 9W is also one side of a reaction zone around tabular dunite. (Dunite-harzburgite contact at 0; plagioclase-in at about 70 cm). In sample series TP90-9 and 9W, the centre of the reaction zone is not at zero, the dunite-harzburgite contact, but within the dunite (negative values). Also shown are data for two plagioclase lherzolite samples within 5 to 10 m of reaction zones, and for three plagioclase lherzolite samples more than 100 m from known reaction zones. The latter are essentially identical to neutron activation analyses of Trinity plagioclase lherzolite²¹ shown in the box to the right. Solid symbols are averages for one sample, and associated error bars are for one standard deviation from the mean.

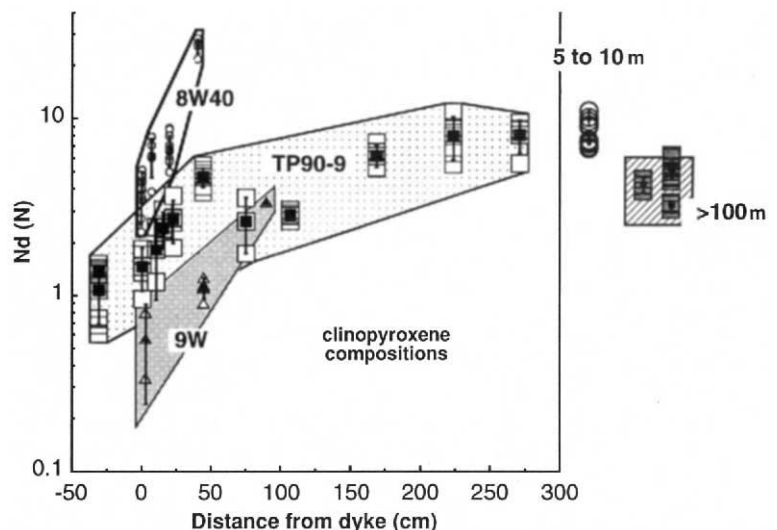


TABLE 1 Values used in modelling

Crystal-liquid distribution coefficients											Chondrite-normalized trace element compositions				
Element	olivine/liq	opx/liq	cpx/liq	spinel/liq	garnet/liq	pyrolite	spinel lherz melt mode	harz reaction product	garnet pyrolite	garnet lherz melt mode	garnet harz reaction product	Figs 2 and 5 Initial liquid reactant	Fig. 2 lherzolite reactant. Residue, 4% melting spinel pyrolite	Fig. 5 pyrolite reactant	Fig. 5 lherzolite reactant. Residue, 20% melting garnet pyrolite
Ce	0.00001	0.003	0.13	0.0006	0.008	0.0238	0.0892	0.009	0.0142	0.0423	0.0016	1.0	0.2790	1.65	0.0460
Nd	0.00007	0.009	0.25	0.0006	0.057	0.0467	0.1731	0.0028	0.0361	0.0996	0.0080	2.0	0.7338	1.78	0.1333
Sm	0.0007	0.02	0.45	0.0006	0.217	0.0854	0.3134	0.0065	0.0885	0.2285	0.0272	5.5	1.2550	2	0.3529
Eu	0.00095	0.03	0.5	0.0006	0.45	0.0971	0.3524	0.0097	0.1411	0.3439	0.0531	6.0	1.3347	2	0.5311
Gd	0.0012	0.04	0.5	0.0006	0.9	0.0999	0.3578	0.0128	0.2319	0.5364	0.1008	5.9	1.3526	2	0.7676
Ti	0.015	0.15	0.4	0.15	0.6	0.1304	0.3785	0.0589	0.1889	0.4006	0.1073	5.7	1.5035	2	0.7044
Dy	0.004	0.06	0.51	0.0015	2	0.1086	0.3746	0.0207	0.4551	1.0095	0.2176	5.5	1.4017	2	1.1174
Er	0.009	0.07	0.52	0.003	3.5	0.1156	0.3849	0.0272	0.7570	1.6507	0.3734	5.3	1.4370	2	1.3619
Yb	0.023	0.1	0.52	0.0045	7	0.1305	0.3956	0.0456	1.4615	3.1412	0.7400	5.0	1.5026	2	1.6129
Phase proportions															
olivine						46.40	44.64		53.54	10.37			50.20		64.33
opx						27.67	55.34	30.00	16.60	17.70	25.00		26.51	16.60	16.33
cpx						17.66	67.22	0.00	9.32	29.54	0.00		15.59	9.32	4.27
spinel						7.51	22.20	2.50	0	0	0		6.90	0	0
garnet						0	0	0	19.77	42.39	10.00		0	19.77	14.12

'harz' is harzburgite.

in most of the upper mantle), so we have omitted it from the modelling presented below.

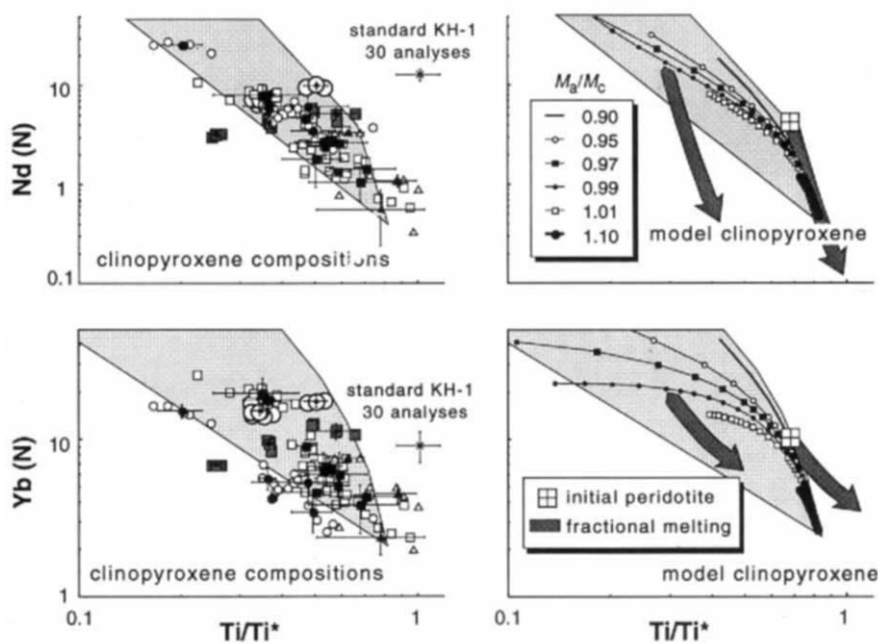
Converting lherzolite to harzburgite

Reaction between ascending melts and surrounding upper-mantle wall rocks will commonly result in dissolution of cpx, and production of a modified liquid undersaturated in cpx^{9,12,14,16} (Fig. 3, bottom left). Small 'y' symbols illustrate the composition of mantle melts formed at pressures from ~30 kbar (richest in ol and cpx components) to ~10 kbar (furthest from ol). The position of these points is based on experimental partial melt compositions for mantle lherzolite (Fig. 3, top and middle left).

Liquids formed at high pressure in the mantle are saturated only in olivine at lower pressure, and will dissolve pyroxenes during melt/rock reaction. Where mantle peridotite is slightly colder than ascending liquid, the liquid will cool slowly during

infiltration and reaction. The shaded regions in Fig. 3, lower left, enclose likely temperature-composition paths for liquid and solid products under these conditions. Initially, liquid saturated only in olivine reacts with peridotite to produce dunite and increasingly opx-rich liquid compositions. As a result, the modified liquid will become saturated in opx. This will occur when the integrated solid/liquid ratio exceeds about 5 in the example illustrated, and at lower solid/liquid ratios where the difference between pressure of melting and pressure of reaction is smaller. At constant temperature or constant enthalpy, reaction between opx-saturated liquid and lherzolite may continue indefinitely, dissolving cpx and precipitating opx and ol to produce harzburgite at nearly constant liquid mass¹⁴. If the liquid is cooling, as illustrated here, it will decrease in mass, producing harzburgite by reaction with lherzolite, and evolving to cpx saturation. If liquid continues to infiltrate through lherzolite after cpx saturation, it will form pyroxene-rich

FIG. 2 Titanium/REE ratio (Ti/Ti^*) against normalized neodymium ($Nd(N)$) and ytterbium ($Yb(N)$) concentrations in cpx, normalized to C1 chondrites⁴⁶. The Ti/Ti^* notation similar in intent to the Eu/Eu^* notation for 'europium anomalies', was adopted by Salters and Shimizu⁴⁷ in work on trace element contents of mantle cpx. Ti/Ti^* should be equivalent to the ratio $Ti(N)/Tb(N)$, where Tb (terbium) is the rare earth element that has the same position as Ti on a compatibility diagram⁴⁹, based on the relative abundance of incompatible trace elements in mid-ocean-ridge basalts. Thus, in 'normal' MORB, $Ti/Ti^* = 1$. Where Ti/Ti^* is < 1 , $Ti(N)$ lies below $Tb(N)$ on the trend formed by chondrite-normalized REE concentration arranged in order of increasing atomic number. Tb concentration is rarely measured, so we use the average value for adjacent rare earths (Dy and Eu) as a proxy for Tb. Where Eu abundance is unknown or close to detection limits, we use the weighted average of Dy and Sm as a proxy for Tb. For our ion-probe data, Ti/Ti^* is calculated as $4Ti(N)/[3Dy(N) + Sm(N)]$. In subsequent diagrams, Ti/Ti^* has also been calculated from literature data as $Ti(N)/Tb(N)$ and $2Ti(N)/[Dy(N) + Eu(N)]$. Diagrams on the left present data from reaction zones in the Trinity peridotite. Symbols for different samples as in Fig. 1. Also shown is an average of 30 analyses of cpx standard KH-1, made over a period of two years. Diagrams on the right are results of modelling in which lherzolite reacts with liquid to form harzburgite plus modified liquid; the models use compiled distribution coefficients²³ and



DePaolo's²⁵ model for combined assimilation and crystal fractionation. Modelling parameters are given in Table 1. Also shown are two trends calculated for fractional melting^{48,50}.

herzolite products with greatly modified trace element concentrations.

Adopting the computational methods of DePaolo²⁵ and solid/liquid distribution coefficients of Kelemen *et al.*²³, we have constructed a simple model in which liquid reacts with herzolite to produce harzburgite. The calculations model the composition of liquid and solid products as a function of increasing reaction progress in which a single 'batch' of liquid reacts with larger and larger masses of herzolite, producing modified liquids and solids. To make a semi-quantitative model of the Trinity reaction zones, we selected an initial liquid with trace element characteristics similar to those inferred from cpx compositions at the centres of reaction zones, and a peridotite reactant with trace

element characteristics like those of Trinity plagioclase herzolite far from reaction zones (Table 1).

The diagrams on the right in Fig. 2 illustrate the results of modelling, which reproduce the trend of the Trinity data. The curves are for varying ratios of solid reactants to solid products, mass assimilated to mass crystallized (M_a/M_c). The shaded fields enclose possible mixtures of reactant and product cpx for M_a/M_c from 0.9 to 0.97, with liquid mass from 100 to 10% of initial liquid mass. The reaction-zone data is best modelled by a reaction process in which liquid mass decreases by 0.03 grams per gram of herzolite reactant ($M_a/M_c = 0.97$). For comparison, we also give the results of fractional melting calculations. We used two initial solid compositions, the Trinity plagioclase herzolite

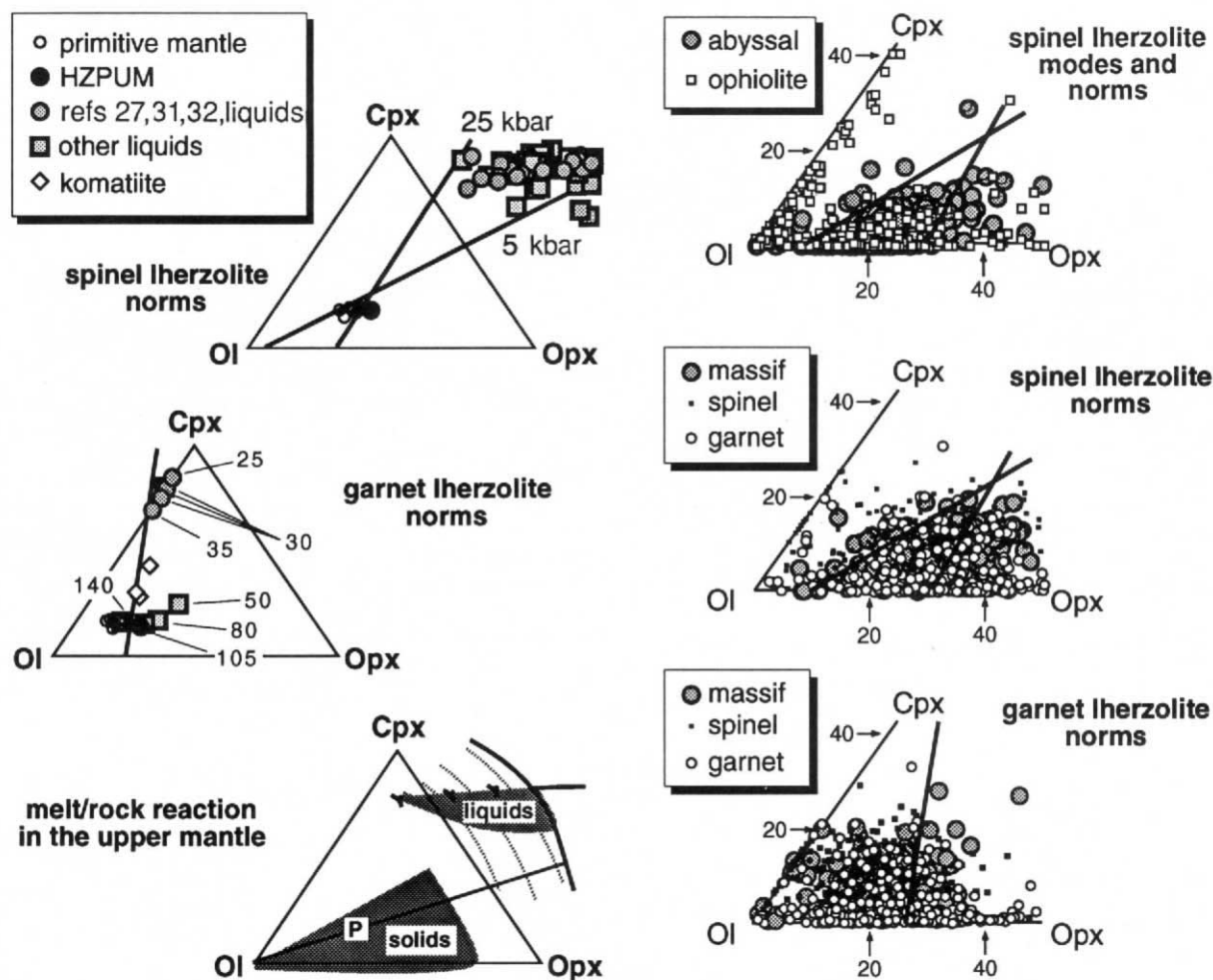


FIG. 3 Possible solid and liquid products of partial melting and magma-mantle interaction compared to compositions of upper-mantle peridotites. Points are norms calculated in terms of wt% ol, opx, cpx and spinel or garnet, projected from spinel or garnet onto the ol-opx-cpx plane. In all compositions molar $\text{Fe}^{2+}/\text{Fe}^{3+}=9$. Except for Na and K, which form (Na,K)-(Al, Cr, Fe^{3+}) Si_2O_6 in cpx, pyroxenes are (Ca, Mg, Fe) Si_2O_6 mixtures. Cpx has 40 mol% $\text{Ca}_2\text{Si}_2\text{O}_6$, and opx 3 mol%, unless one or the other is absent, in which case more calcic cpx or less calcic opx are formed. All other trivalent cations in spinel or garnet. Garnet has 10 mol% $\text{Ca}_3\text{Al}_2\text{Si}_3\text{O}_{12}$ unless opx or cpx is absent, in which case more or less calcic garnet is formed. In addition to spinel and garnet herzolite norms, we calculated 'Tscheramak's norms' with all trivalent cations in pyroxenes. Although Tscheramak's norms for liquids have more ol and less pyroxene than spinel herzolite norms (because of the reaction $\text{MgAl}_2\text{O}_4 + 2\text{MgSiO}_3 = \text{MgAl}_2\text{SiO}_6 + \text{Mg}_2\text{SiO}_4$, for example), they yield ol-opx-cpx proportions for residua of partial melting similar to those obtained using spinel herzolite norms (maximum of ~26% opx along the opx-ol join). For 816 massif and xenolith peridotites, Tscheramak's norms differed from spinel herzolite norms by 1.5 ± 1.1 (1 σ) wt% olivine. Upper and middle left, compositions of liquids reported to be in equilibrium with herzolite at upper-mantle pressures^{27,31-37}. Heavy solid

lines delineate solid residua which can be produced by removing partial melt compositions from nine primitive mantle compositions²³⁻²⁵. Also shown are three komatiite compositions⁷, and the primitive upper-mantle composition of Hart and Zindler³⁸, labelled HZPUM. Lower left, schematic illustration of solid and liquid compositions produced by reaction between melt, produced at relatively high pressure, and solid mantle peridotite at a lower pressure. P, primitive mantle herzolite. Dark lines show the boundaries of olivine and pyroxene primary phase fields at ~10 kbar and 'Y' symbols show the composition of partial melts of herzolite at higher pressures, from the upper two diagrams. Dashed lines schematically illustrate isotherms on the liquidus surface in the olivine field at ~10 kbar. All compositions plotted with spinel herzolite norms. Right, wt% modes (398 abyssal peridotites and 254 ophiolite peridotites, recalculated plagioclase-free) and norms (129 ophiolite peridotites, 138 massif peridotites from Beni Boussera, Ronda, Pyrenees, Lanzo, Massif Central, Lizard, 377 garnet-free, spinel-bearing mantle xenoliths, and 301 garnet-bearing mantle xenoliths) of upper-mantle peridotites (>40% olivine). Data from refs 2-4, 6, 8, 11, 28 with McDonough's associated data base, 29, 30, 39, 51-58, R. G. Coleman (personal communication) and our unpublished work.

far from reaction zones, and a lherzolite from within a reaction zone. Fractional melting produces less variation of Ti/REE with Nd concentration than is observed in our samples. This is also true for batch melting (not shown).

Field observations in the Trinity peridotite and elsewhere demonstrate that harzburgite can be produced in an open system by reaction between lherzolite and migrating basaltic melts. If this process always increased magma mass, it would be similar to closed-system partial melting, and the interpretation of harzburgites as depleted residua would not be in question. But the increase in incompatible element concentration as the reaction progresses, modelled by a process in which magma mass decreases during reaction, illustrates that not all harzburgites are depleted in mass relative to parental lherzolites.

The harzburgite problem

If harzburgite can form by reaction between lherzolite and ascending basaltic liquid, this raises the question of how much of the mantle may have been affected by such a process. As a preamble to further modelling, we formulate the 'harzburgite problem'. Simply stated, many samples derived from the sub-continental mantle are too opx-rich to be residua of partial melting from proposed primitive mantle compositions based on the composition of C1 chondrites²⁶⁻²⁸. By contrast the major and trace element composition of most abyssal peridotites can be explained as the residual of partial melting^{2-4,29,30}.

Figure 3 illustrates this point. The two upper diagrams on the left summarize data on melts reported to be in equilibrium with lherzolite at upper mantle pressures^{27,31-37}. Points are norms calculated in terms of opx, cpx, ol, and spinel or garnet, and are projected from spinel or garnet onto the ol-opx-cpx plane. Liquids plotted with spinel lherzolite norms are from experiments at 5 to 25 kbar. Liquids with garnet lherzolite norms are from 25 to 140 kbar, and are labelled with experimental pressure in kilobars. We also plot three komatiite compositions (from ref. 7) as they are possible high-pressure partial melts. We use norms, rather than oxides, to interpret results in terms of the phase equilibria of melting and melt/rock reaction. We project from spinel or garnet, rather than plag, because plag is not a stable phase in most of the upper mantle. Heavy solid lines delineate solid residua which can be produced by removing partial melt compositions from primitive mantle compositions similar to pyrolite (ref. 26, and summaries in refs 27 and 28). For both spinel and garnet lherzolite norms, the vectors define an upper limit for modal opx in the residua of partial melting

from these primitive mantle compositions, ending at 25–30% opx along the opx-ol join.

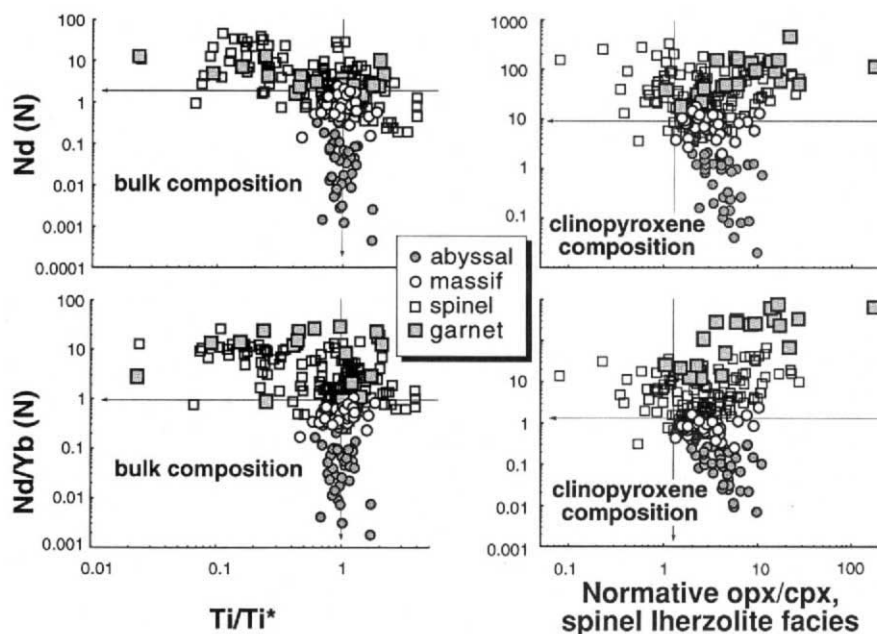
Diagrams on the right illustrate the composition of more than 1,000 mantle peridotite samples (>40% ol). Superimposed on these data are the heavy solid lines from the upper left-hand diagrams, illustrating limits for residual compositions. Whereas most abyssal and ophiolite peridotites (>95% of 398 abyssal samples) could be residual, more than 25% of massif and xenolith peridotites have opx contents and opx/cpx ratios higher than possible in the residua of partial melting. Instead, many may have formed by melt/rock reaction. (Peridotites that do plot within the range of possible residual compositions could be residual, but might also have formed by magma-mantle interaction).

Figure 4 summarizes available trace element data for the same groups of mantle samples. We plot both Nd concentration and Nd/Yb ratio to illustrate systematics of light and heavy REE distribution. We view these as a function of opx/cpx and Ti/REE in order to investigate the possible role of melt/rock interaction. Abyssal peridotites have chondritic Ti/REE, high opx/cpx and Nd/Yb less than chondritic, consistent with a residual origin. No other peridotite suite in our compilation shares these characteristics. Instead massifs and xenoliths, and our Trinity samples (compare Fig. 2), generally have sub-chondritic Ti/REE, opx/cpx greater than the primitive mantle and Nd/Yb greater than chondritic.

Two possible explanations for the major element data are that high-pressure partial melts are sufficiently olivine-rich and opx-poor to enrich the residual mantle in opx, or that the primitive upper mantle is considerably more SiO₂-rich (and therefore opx-rich) than C1 chondrite based primitive mantle compositions. Boyd⁷ noted that many garnet peridotite xenoliths from the Kapvaal craton were more opx-rich than other mantle samples, with an average of ~31 wt% opx. He explained their composition as a consequence of high degrees of partial melting of primitive mantle at high pressure, producing komatiitic liquids and very cpx-poor, opx-rich residua. By contrast, our calculations suggest that komatiites, and experimentally produced high-pressure melts, are too opx-rich to produce harzburgite with more than 26 wt% opx as a residue of melting from pyrolite.

Hart and Zindler's³⁸ primitive mantle composition (HZPUM), based on the composition of mantle samples and chondritic element ratios, is more opx-rich than pyrolite. This may reflect a more SiO₂-rich composition for the primitive upper mantle

FIG. 4 Normative and trace element compositions of mantle peridotites. Norms as in Fig. 3. Nd(N), Yb(N), and Ti/Ti* as in Fig. 2. Where cpx or opx is absent, opx/cpx calculated as opx/(0.01) or (0.01)/cpx. REE and Ti in abyssal peridotite bulk compositions, and in massif and xenolith cpx, have been calculated using the distribution coefficients summarized in Table 1 and normative or modal mineral proportions. Arrows show the approximate composition of the primitive mantle^{26,28,38}.



than can be inferred from C1 chondrites. Conversely, it may reflect a systematic enrichment of sub-continental mantle in SiO_2 , even in the rare samples that have chondritic major and trace element ratios. In any case, adoption of their estimate for the primitive upper mantle would leave the origin of samples with >32 wt% opx unexplained. Primitive mantle compositions more opx-rich than Hart and Zindler's have been proposed (summarized in ref. 28). Partial melting of these could produce harzburgite residue with more than 32 wt% opx.

Any melting model must also account for the trace element composition of mantle samples. Whereas partial melting should generally produce sub-chondritic Nd/Yb ratios, for example, opx-rich mantle xenoliths commonly have Nd/Yb ratios much greater than the chondritic value (Fig. 4). This observation, coupled with the axiom that harzburgites are residua of melting, resulted in the belief that major and trace element characteristics of most mantle xenoliths are decoupled; that is, produced by two distinct processes. In one such scheme³⁹, much of the mantle has first been depleted in basaltic major and trace components by melting, and later enriched in incompatible trace elements by metasomatism.

There is good reason to believe that much of the mantle has been depleted by partial melting. Long-term enrichment of the crust and depletion of the mantle in incompatible elements is implied by global trace element and radiogenic isotope systematics⁴⁰. But in rocks modified by melt/rock reaction, radiogenic isotope ratios may largely reflect the history of the source region for the liquid reactants, and we must look elsewhere to discover the history of the rock. Major element systematics in ancient residual peridotites should be qualitatively similar to those in abyssal peridotites. Yet abyssal peridotites have lower opx contents than many ophiolite peridotites and mantle xenoliths (Figs 3 and 4). We tentatively conclude that the high opx contents and high opx/cpx ratios of many mantle samples, as well as their high Nd/Yb ratios, cannot be explained as a consequence of partial melting processes.

Instead, we propose that both major and trace element characteristics of lithospheric mantle samples were produced by magma-mantle interaction. Some simple modelling is presented in Fig. 5. Solid reactants are garnet peridotites, and solid products are harzburgites with and without 5 wt% garnet. Interaction produces light REE enrichment as a function of reaction progress. Whereas the $D(\text{Ti})$ between opx, ol, and spinel and basaltic liquid is greater than coefficients for the REE, $D(\text{Ti})$ between garnet and liquid is less than for the REE^{41,42}. Thus, Ti/REE in derivative liquids and solids is nearly constant when garnet, opx and ol are produced during reaction, and decreases as a function of reaction progress when opx and ol are produced without garnet. We use the same liquid reactant as previously, and two compositions for the solid reactant: pyrolite, and the depleted residue of 20% partial melting of pyrolite in garnet lherzolite facies. In using pyrolite, we do not wish to imply that large portions of the mantle retain primitive compositions. We simply use different endmembers for illustration. A previously depleted solid reactant may be required to produce very low Ti/REE at moderate Nd/Yb as observed in some xenoliths.

Discussion

Interaction between ascending magma and mantle wall rock can produce dunite, then harzburgite, and finally pyroxene-rich lherzolite, even when the magma mass is decreasing. Therefore the axiom that harzburgites represent the depleted residua of partial melting cannot be accepted without tests. Many mantle harzburgites and opx-rich peridotites cannot be the residua of partial melting from a primitive mantle; instead, their composition can be accounted for by magma-mantle interaction involving precipitation of opx and ol, sometimes with garnet, under conditions of gradually decreasing magma mass.

Magma-mantle interaction is likely where melts migrate upward by intergranular flow. Although it may occur beneath

oceanic spreading centres, the geochemical effects are likely to be absent or subdued where rapid decompression of the entire system leads to increasing magma mass. On the other hand, the effects will be enhanced where temperatures are below the lherzolite solidus, for example in cold, static lithosphere beneath continental cratons. Magma generation in the aesthenosphere below cratons may be slow, however, and few liquids will actually penetrate the entire lithosphere. In this context, subduction-related magmatic arcs may be transitional between the spreading ridges and and cratonic settings. A lower average pressure of interaction, a smaller solid/liquid ratio, less-modified mantle compositions, and more commonly observed liquid products should characterize magma-mantle interaction in arcs. As proposed previously^{9,14-18,23} many of the major and trace element characteristics that distinguish subduction-related magmas from mid-ocean-ridge basalts may be explained as the result of magma-mantle interaction. Sub-arc and sub-continental mantle should not necessarily be viewed as depleted. Instead, they may be enriched by a melt/rock reaction process transporting silica and incompatible trace elements from deeper in the mantle into the lithosphere.

Our modelling differs from previous treatments of melt/rock reaction in the upper mantle (for example refs 43-45) in one main respect: we account for variation in the mass and major element composition, as well as trace element composition, of product solids and liquids. In addition, we monitor the composition of a single batch of liquid migrating through previously unaltered peridotite, rather than the composition of a volume of peridotite reacting with a liquid of constant initial composition. Finally, we do not attempt to account for the effects of solid or liquid diffusion in our calculations. We hope that our

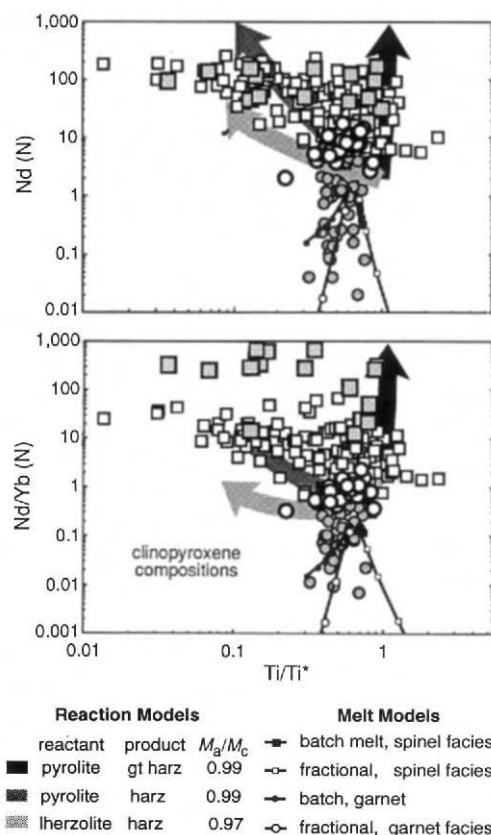


FIG. 5 Results of modelling magma-mantle interaction dissolving cpx and producing opx plus ol, with or without garnet, converting lherzolite to harzburgite. Modelling parameters are given in Table 1. Also shown are model results for batch and fractional partial melting^{49,50}. Results are superimposed on mantle compositions, with symbols as in Fig. 4.

hypothesis will stimulate continuing investigation. The stoichiometry of magma-mantle interaction in plagioclase and spinel peridotite facies has been roughly predicted by Kelemen⁷, but for these reactions (and particularly for reactions in garnet peridotite facies) there can be no substitute for improved silicate liquid solution models and a larger experimental data base. Similarly, data on trace element exchange equilibria as a function of temperature, pressure and composition, and on multi-

component diffusion of major and trace elements in solids and liquids, are sorely needed. There are additional difficulties with the theory and computation of the kinetics of magma-mantle interaction. But wherever the primary causes of compositional variation in derivative liquids and solids are solution and precipitation reactions, rather than solid diffusion mechanisms, local equilibrium models such as ours provide a realistic outline for future investigation. □

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Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II

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Phosphorylation of the carboxy-terminal domain of the largest subunit of RNA polymerase II is believed to control the transition from transcription initiation to elongation. The general transcription factor IIH (TFIIH) contains a kinase activity capable of phosphorylating this domain. Factors that promote the association of RNA polymerase II with the preinitiation complex stimulate this activity. The transcription factor IIE, which is required for the stable association of TFIIH with the preinitiation complex, affects the processivity of TFIIH kinase.

INITIATION of transcription by RNA polymerase II is a complex process requiring, in addition to the polymerase itself, seven auxiliary factors¹. Entry of the polymerase into the transcription cycle is mediated by transcription factor TFIIF (ref. 2) and requires a DNA-protein complex composed of the TATA-binding protein subunit of TFIID in association with the TATA motif and TFIIB (DB complex)^{3,4}. The largest subunit of mam-

alian RNA polymerase II contains a heptapeptide repeat (YSPTSPS; single-letter amino-acid code) occurring 52 times at its C terminus⁵. Because the heptapeptide contains serine, threonine and tyrosine, it is prone to phosphorylation. As a result, RNA polymerase II occurs in two forms *in vivo*, a highly phosphorylated IIO form and a non-phosphorylated IIA form⁵. The non-phosphorylated form of RNA polymerase II is recruited by TFIIF to the DB complex, producing the DBPolIF complex⁶. This complex is then recognized by TFIIE, TFIIF

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and TFIIJ, which enter the transcription cycle in that order, to generate a transcription-competent complex⁷. Although the IIA form of RNA polymerase II enters the transcription cycle, elongation is catalysed by the phosphorylated (IIO) form^{8,9}, suggesting that the polymerase is phosphorylated in the preinitiation complex. Because the IIA, but not the IIO form of RNA polymerase II interacts with the TATA-binding subunit¹⁰, we speculated that phosphorylation of RNA polymerase IIA in the preinitiation complex causes a conformation change resulting in an elongation-competent form of the complex^{10,11}.

The search for factors phosphorylating the C-terminal domain (CTD) has resulted in the identification of many protein kinases¹²⁻¹⁹. It is puzzling that so many protein kinases have been identified. One explanation is that most kinases are 'promiscuous' *in vitro*, owing to the removal of kinase-regulatory molecules. Alternatively, a variety of CTD-kinases may be necessary to modulate the different roles that this repeat is thought to play in the transcription cycle. For example, the CTD not only participates in regulating the transition from initiation to elongation²⁰, but also has been implicated in mediating the response to certain activators in yeast²¹. Here we have analysed the protein kinase(s) mediating phosphorylation of the CTD of RNA polymerase II during basal transcription. We show that phosphorylation of the CTD is mediated by the general transcription factor TFIIH.

Association of TFIIH and preinitiation complex

Initiation of transcription is preceded by the highly ordered assembly of the general transcription factors and RNA polymerase II into a functional preinitiation complex on promoter-containing DNA¹. Preinitiation complex intermediates can be produced *in vitro* and visualized on non-denaturing polyacrylamide gels using the gel mobility shift assay⁴. To analyse the effect of ATP on the different intermediates during formation of a transcription-competent complex, different complexes were formed in the presence or absence of ATP. ATP was without effect on the migration of the DB complex containing TFIIA (DAB)³, the DABPoIF and DABPoIFE complexes (Fig. 1, lanes 1-6). The migration on a non-denaturing gel of a complex containing TFIIH-DABPoIFEH was, however, retarded in the presence of ATP (compare lanes 7 and 8). This effect was not specific for ATP, because GTP or the deoxy analogues could retard the mobility of the DABPoIFEH complex (data not shown, and ref. 6). The RNA polymerase II used in the analysis was the nonphosphorylated IIA; however, the enzyme recovered from the shifted complex comigrated with the IIO form on a polyacrylamide-SDS gel, as detected by western blot analysis using antibodies directed against exon 5 of the largest subunit

of RNA polymerase II (ref. 22, and data not shown; for details see ref. 6 and below). Thus, the nucleotide-induced slower migration of the complex containing TFIIH was due to phosphorylation of RNA polymerase II. These studies indicate that the association of TFIIH with the preinitiation complex results in a multiprotein complex capable of phosphorylating the polymerase.

TFIIH phosphorylates RNA polymerase II

To investigate whether TFIIH could phosphorylate RNA polymerase II in the absence of the other factors required for basal transcription, polymerase IIA and TFIIH were incubated with [γ -³²P]ATP under DNA-binding conditions and the products analysed by electrophoresis on a polyacrylamide-SDS gel. Under these conditions, a low level of label was incorporated into RNA polymerase II (Fig. 2a, lane 5). The labelling of this enzyme was specific for TFIIH as the incubation of polymerase IIA (lane 1), or TFIIH (lane 2), or all the factors required for transcription except TFIIH, in either the absence (lane 3), or presence (lane 4) of polymerase IIA, resulted in no detectable labelling of the largest subunit of RNA polymerase II. TFIIH-mediated phosphorylation of polymerase IIA was stimulated by the TATA-binding subunit (lane 6), and further by the subsequent addition of TFIIB (lane 7) and TFIIF (lane 8). The stimulation by TFIIB and TFIIF was dependent on the TATA-binding protein (data not shown). The addition of TFIIE to the assay resulted in both a quantitative (fourfold) and qualitative stimulation of phosphorylation of RNA polymerase II, as most of the label was incorporated into the IIO form of the enzyme (lane 9). The addition of TFIIJ, in the presence of TFIIE, further stimulated the production of the IIO form of the polymerase (lane 10). The stimulation of polymerase phosphorylation resulting from the addition of TFIIE may be related to the observation that TFIIH can stably associate with the complex only after entry of TFIIE into the DBPoIF complex⁷. This suggested that TFIIE may stimulate the processivity of TFIIH-kinase activity. This was further investigated by analysing the extent of phosphorylation and the form of RNA polymerase II recovered as a function of time in the presence or absence of TFIIE.

As shown in Fig. 2b, in reactions containing TFIIE, the IIO form of RNA polymerase II predominates as the labelled product after just 5 min incubation (lane 6). When TFIIE is omitted from the reaction, a heterogeneous array of phosphorylation products distributed between the IIA and IIO forms of the polymerase persists even after 90 min incubation (lanes 1-5). This is also shown in Fig. 2c, in which the total amount of radiolabel incorporated into RNA polymerase II (IIA and IIO) was compared with that incorporated into the IIO form. Total incorporation of ³²P parallels conversion to the IIO form of the polymerase in reactions containing TFIIE, whereas at best only 40% of IIO form could be detected in reactions in the absence of TFIIE. These results indicate that the TFIIH-associated kinase activity acts in a processive fashion in the presence of TFIIE.

TFIIH phosphorylates the CTD

Having observed that RNA polymerase II is phosphorylated by TFIIH, we next investigated whether the CTD was being phosphorylated specifically. We used RNA polymerase IIB (ref. 23), which contains no CTD, in a kinase assay analogous to that shown in Fig. 2. If TFIIH specifically phosphorylates the CTD, the TFIIH kinase would not be expected to be able to phosphorylate the IIB polymerase. As shown in Fig. 3a (lanes 5-7), no phosphorylation of the polymerase was detectable when RNA polymerase IIB was used as a substrate. RNA polymerase IIO was also phosphorylated by TFIIH (lanes 8-10), but the extent of phosphorylation was about 10-fold lower than for the IIA polymerase (compare lanes 8-10 with 2-4, respectively). Because association of RNA polymerase II with the preinitiation complex stimulates kinase activity, this is probably related to

FIG. 1 Effect of ATP on transcription complexes intermediates. DNA-binding reactions were done with and without ATP (1 mM) on a 3' end-labelled DNA fragment containing the Ad-MLP (-40 to +13), as previously described⁷. Factors added were as follows and are indicated at the top of the panel. D indicates yTBP³ (S-Sepharose, 40 ng), recombinant TFIIB³³ (phosphocellulose, 10 ng), rTFIIE³⁴ (Sepharacyl 200, 30 ng), TFIIF³⁵ (TSK-phenyl, 90 ng), RNA polymerase II (RNA Pol II)⁶ (DEAE-5PW, 110 ng), TFIIH⁷ (hydroxylapatite, 45 ng), TFIJ⁷ (mono-S, 200 ng). Products were separated on a native polyacrylamide gel as previously described. TFIIA was used only in this experiment. Cortes *et al.* have shown that TFIIA is not essential for basal transcription when recombinant TBP is used²⁹.

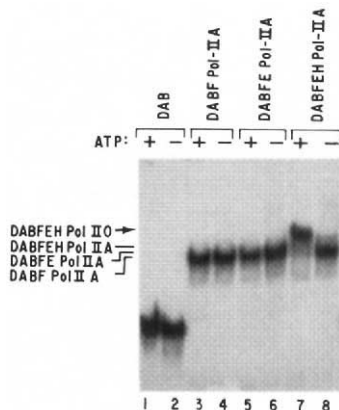
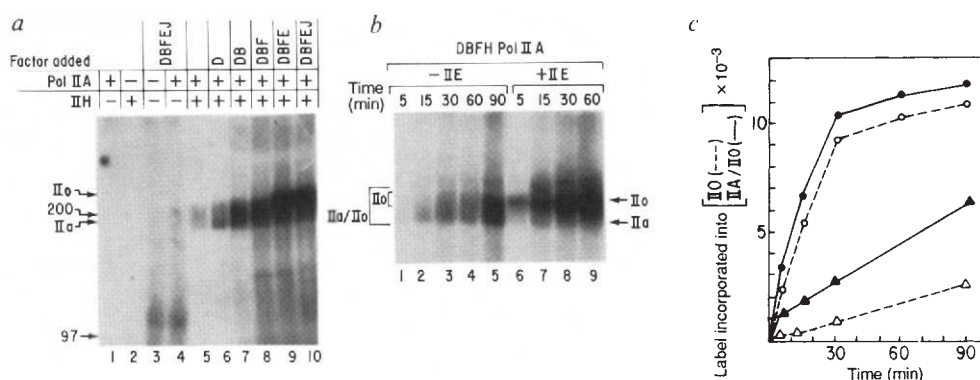


FIG. 2 *a*, TFIH phosphorylates RNA Pol IIA. Phosphorylation reactions were done under DNA-binding conditions (those described in Fig. 1). Reaction mixtures (25 μ l) contained, besides proteins, 10 mM HEPES, pH 7.9, 4 mM MgCl₂, 60 mM KCl, 5 mM (NH₄)₂SO₄, 8% (v/v) glycerol, 2% (w/v) polyethylene glycol 8000, 2 mM β -mercaptoethanol, 0.1 mM EDTA, 20 ng μ l⁻¹ poly(dI-dC) · poly(dI-dC), 40 μ M [γ -³²P]ATP and 2 ng nonlabelled DNA fragment containing a sequence of Ad-MLP (-40 to +13). Factors added are indicated at the top of the panel. Products of the reaction were separated on a 5.5% polyacrylamide-SDS gel. *b*, *c*, Effect of TFIIIE on TFIH-mediated phosphorylation of RNA Pol IIA. Kinase reactions were done in the presence and absence of TFIIIE, as indicated at the top of *b*. Assay conditions were as described for *a* and contained transcription factors γ TBP, IIB, IIF, IIH and RNA Pol IIA. Reactions were incubated for different periods of time as indicated. Products were separated by electrophoresis on a 5.5% polyacrylamide-SDS gel.



Quantification of the amount of ³²P incorporated into RNA Pol IIO and RNA Pol IIA/IIO was done using a phosphorimager. The section of the gel used for quantification of the IIO and IIA/IIO forms of RNA Pol II is indicated on the left of the panel. The values obtained are graphically represented in *c*. Circles and triangles denote reactions done in the presence or absence of TFIIIE, respectively.

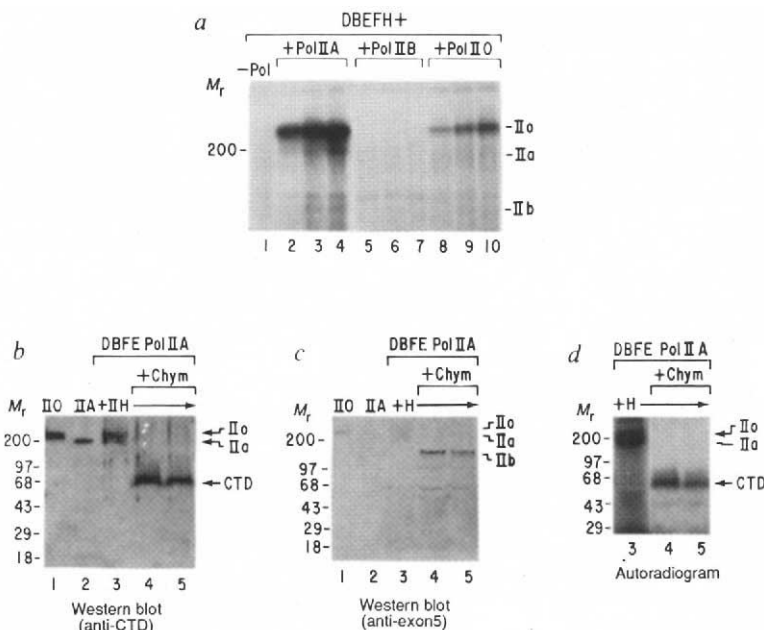
the observation that polymerase IIO associates less efficiently with the preinitiation complex than polymerase IIA⁶. In an analogous experiment, we found that when polymerase IIB was incorporated into a reconstituted system containing highly purified factors, basal transcription remained TFIH-dependent (data not shown). Thus, the role of TFIH in transcription is not solely to mediate the transition from initiation to elongation by phosphorylating the CTD.

The fact that RNA polymerase IIA (and, to a lesser extent, RNA polymerase IIO), but not RNA polymerase IIB was used as a phosphorylation substrate, strongly suggested that the CTD was the specific target of the TFIH kinase. To demonstrate this conclusively, kinase reactions were done as described for Fig. 2 and followed by chymotrypsin digestion, which cleaves between the CTD and the body of the largest subunit of RNA polymerase II²⁴. Products of the reactions were separated by electrophoresis on a polyacrylamide-SDS gel and transferred to a nitrocellulose membrane. Western blot analysis using monoclonal antibodies directed against the CTD²⁵ demonstrated that incubation of polymerase IIA with TFIH, in the presence of TATA-binding protein, TFIIB, TFIIF and TFIIIE, gives rise to

a form of RNA polymerase II comigrating with the phosphorylated IIO form of the enzyme (Fig. 3*b*; compare lane 3 with lanes 1 and 2). Digestion of the *in vitro* phosphorylated RNA polymerase II with chymotrypsin generated two products. One of these was specifically detected with antibodies against the CTD²⁵ and migrated on the denaturing gel with an *R_f* value corresponding to an *M_r* of about 60K (Fig. 3*b*, lanes 4 and 5). The other product, which migrated on the gel with an *R_f* value corresponding to about 180K, was detected with antibodies against exon 5 of the largest subunit of RNA polymerase II²², but not with the anti-CTD antibodies (compare lanes 4 and 5 between Fig. 3*b*, *c*). Thus the 60K and 180K polypeptides correspond to the CTD and the CTD-less IIB subunit of RNA polymerase II, respectively. After chymotrypsin digestion, all of the label was found in the 60K polypeptide and none could be detected in the IIB subunit (Fig. 3*d*, lanes 4 and 5). Thus, these results demonstrate that TFIH phosphorylates the CTD. Phosphoamino-acid analysis reveals that serine (90%) and, to a lesser extent, threonine (10%) residues are phosphorylated (data not shown).

Our results so far indicate that RNA polymerase IIA can be

FIG. 3 TFIH phosphorylates the CTD. *a*, TFIH-mediated phosphorylation of the different forms of RNA Pol II was done as described in Fig. 2. Reactions contained different RNA Pol IIs, [γ -³²P]ATP, and the general transcription factors, as indicated. Products were separated by electrophoresis on a 5.5% polyacrylamide-SDS gel. *b*, *c*, *d*, RNA Pol IIA was phosphorylated with TFIH and [γ -³²P]ATP in the presence of the other transcription factors, as described above. Two thirds of the reaction was further incubated with chymotrypsin²⁴ (90 ng) for 15 and 30 min at 25 °C. Products of the reaction, along with the untreated sample and RNA Pol IIA and IIO, were separated by electrophoresis on a 5–17.5% gradient polyacrylamide-SDS gel and transferred to a nitrocellulose membrane. *b*, The membrane was reacted with CTD-monoclonal antibodies²⁵ and reacting materials visualized using the enhanced chemiluminescence method. Lanes 1 and 2 indicate the migration of the IIO and IIA subunits of RNA Pol II, respectively. Lane 3 represents the product of the phosphorylation reaction. Lanes 4 and 5 are the products of the phosphorylation reaction followed by chymotrypsin digestion. *c*, After incubation with CTD-monoclonal antibodies, the nitrocellulose membrane was immediately incubated with antibodies directed against exon 5 of the largest subunit of RNA Pol II²². *d*, Autoradiogram of the nitrocellulose membrane containing lanes 3–5 described in *b*.



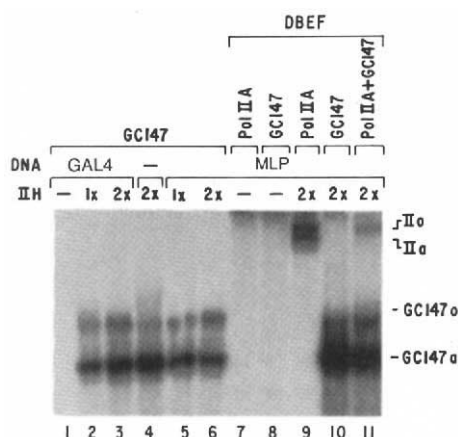


FIG. 4 A CTD-containing peptide is phosphorylated by TFIIF. Phosphorylation reactions were done as described in Fig. 2 and contained TFIIF (hydroxyl-apatite fraction, 22 ng (1x) and 44 ng (2x)), the CTD-containing fusion protein¹⁵ (40 ng), the general transcription factors, RNA Pol II A and different DNA, as indicated in the figure. Products of the reaction were separated on a 9% polyacrylamide-SDS gel.

phosphorylated by TFIIF. But they do not rule out the possibility that TFIIF together with a subunit of RNA polymerase II phosphorylates the CTD. To investigate this possibility, a bacterially expressed fusion protein (GC147)¹⁵ composed of the CTD, the GAL4 DNA-binding domain and glutathione-*S*-transferase was used as substrate in the TFIIF kinase reaction. The incubation of TFIIF with GC147 in the presence of [γ^{32} -P]ATP resulted in phosphorylation of the fusion protein (Fig. 4). This phosphorylation was dependent on TFIIF but was independent of DNA (lanes 1–6). The general transcription factors could also stimulate TFIIF-mediated phosphorylation of GC147 (lane 10). Furthermore, a competition between RNA polymerase II and the fusion protein was observed (lane 11), indicating that TFIIF phosphorylates the CTD both of the fusion protein and of RNA polymerase II. These results demonstrate that TFIIF phosphorylates the CTD and, more importantly, that the general transcription factors can stimulate this reaction independently of RNA polymerase II. The most logical explanation is that the general transcription factors and TFIIF can form a multiprotein complex. The differences between RNA polymerase II and the DNA-independent activity observed with the fusion protein may reflect the structure of the CTD in the proteins (RNA polymerase II versus fusion), as it is possible that association of RNA polymerase II with the preinitiation complex results in unfolding of the tail. Also, a peptide containing five copies of the CTD can be phosphorylated by TFIIF but is an extremely poor substrate (data not shown).

TFIIF-kinase activity stimulated by DNA elements

These results indicate that factors promoting association of RNA polymerase II with the preinitiation complex (TATA-binding protein, TFIIB, TFIIF)⁷, and/or interacting with the CTD (TATA-binding protein)¹⁰, stimulated the extent of RNA polymerase II phosphorylation. Next we investigated whether DNA elements capable of directing formation of a transcription-competent complex (TATA and/or initiator) affect the TFIIF-associated kinase activity. The kinase reactions were done as described for Fig. 2 in the presence of DNA fragments containing the adenovirus major late promoter (Ad-MLP) TATA and initiator, the TATA, the initiator, and random sequences (poly(dI-dC)), respectively. The TATA and/or initiator could stimulate the TFIIF-mediated phosphorylation of RNA polymerase II (Fig. 5). In the presence of nonspecific DNA, which is unable to mediate specific association of RNA polymerase II, the phosphorylation was hardly detected (lane 1 and data not shown).

TFIIF transcriptional and kinase activities

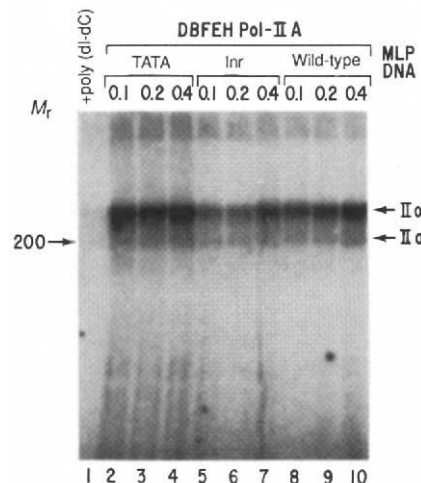
TFIIF is a multisubunit protein composed of polypeptides of M_r 92, 62, 43, 40 and 35K^{7,26}. TFIIF can be purified both from the 0.5 M and from the 1.0 M KCl step washes during phosphocellulose chromatography of HeLa cell extracts⁷. We have found that TFIIF-transcription and kinase activities coeluted through extensive chromatography of both the 0.5 M and 1.0 M KCl-derived TFIIF protein fractions (data not shown). TFIIF was immunologically detected in the same fractions containing transcription and kinase activity using monoclonal antibodies (3c9) raised against the recombinant 62K subunit of TFIIF (BTF2)²⁷ (data not shown). To investigate further whether both transcription and kinase activities were associated with TFIIF, the effect of the antibodies both on transcription and on kinase activities was analysed. The addition of 3c9 to the transcription (Fig. 6a, lanes 4–6) or kinase assays (Fig. 6b, lanes 3–5) inhibited both activities. This inhibition was observed for TFIIF fractions derived from both 0.5 M and 1.0 M phosphocellulose washes (data not shown). The inhibition was specific, as monoclonal antibodies directed against the amino terminus of *Drosophila* TATA-binding protein²⁸ (Fig. 6a, lane 3), or against the protein cdc2 (Fig. 6b, lanes 12–14), were without effect on transcription and kinase activities, respectively. Furthermore, the inhibition of transcription and kinase activities could be overcome by the addition of excess TFIIF (Fig. 6a, lanes 7 and 8, and b, lanes 6–8) or BTF2 which was purified independently²⁶ (data not shown), but not TFIIB (Fig. 6a, lanes 9–11, and b, lanes 9–11). Therefore, these results demonstrate that the transcription and kinase activities are both contained in TFIIF.

Discussion

Our studies have shown that the general transcription factor IIF phosphorylates the CTD of RNA polymerase II as well as a bacterially expressed fusion protein containing the CTD. Although TFIIF can specifically phosphorylate RNA polymerase II in the absence of any other factors, TFIIF-kinase activity was greatly stimulated by factors promoting the association of RNA polymerase II into a preinitiation complex and by DNA elements capable of directing the formation of a transcription-competent complex.

RNA polymerase II phosphorylation by TFIIF occurred on its CTD and was extensive, as we were able to demonstrate complete conversion of the IIA form of the enzyme to the phosphorylated IIO form. The TFIIF-associated kinase activity is a serine/threonine kinase and is highly specific for RNA polymerase II. We were not able to detect TFIIF kinase activity using histone H1 or casein as substrates (data not shown).

FIG. 5 Effect of DNA on TFIIF-mediated phosphorylation of RNAPII. Phosphorylation reactions were done as indicated on Fig. 2a and contained transcription factors as indicated on the figure. The reactions contained only nonspecific competitor DNA poly(dI-dC) (50 ng, lane 1) or poly(dI-dC) plus different oligonucleotides containing either the Ad-MLP-TATA and initiator motifs, the TATA motif, or the Inr motif, as indicated at the top of the panel. Products were separated by electrophoresis on a 5.5% polyacrylamide-SDS gel.



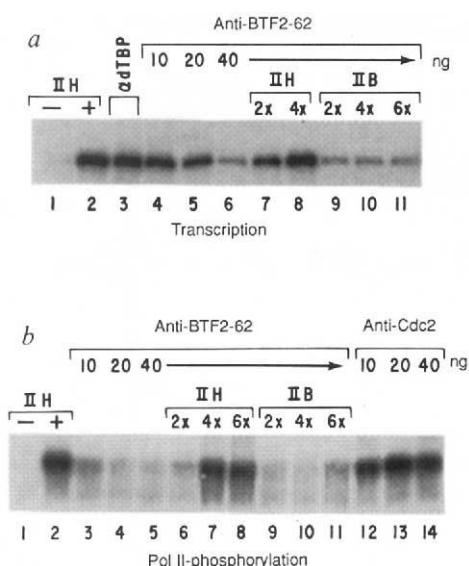


FIG. 6 Monoclonal antibodies against p62 inhibit TFIH-associated transcription and kinase activities. *a*, Transcription reaction mixtures were reconstituted using the general transcription factors and the Ad-MLP (PMLC₂AT)³⁶. TFIH (150 ng) was incubated with antibodies (α TBP²⁸, 40 ng, α BTF2²⁷, as indicated on the panel) for 10 min on ice before addition to the assay. Conditions were as previously described⁷ and contained the following factors: yTBP (S-sepharose, 30 ng), rTFIIB (phosphocellulose, 30 ng), rTFIIE (Sephacryl 200, 30 ng), RNA Pol IIA (DEAE-5PW, 60 ng) or RNA Pol IIB, TFIIF (TSK-phenyl, 90 ng), TFIH (mono-Q, 25 ng), TFIJ (phenyl-superose, 200 ng). Products of the reactions were separated by electrophoresis on a polyacrylamide-urea gel. 2 $\times$ and 4 $\times$ indicate that the amount of the factor added to the assay was increased by two- or fourfold. In this case the excess factor was not incubated with the antibodies. *b*, TFIH-mediated phosphorylation of RNA Pol IIA was done as described in Fig. 2a. Antibodies (as indicated on the panel) were first incubated with TFIH (50 ng) for 10 min on ice. 2 $\times$, 4 $\times$ and 6 $\times$ indicate that the amount of the factor (TFIIF or TFIIB, as indicated) added to the assay was increased by two-, four- or sixfold.

TFIIH (BTF-2)^{7,26} is a multisubunit factor consisting of five polypeptides (92, 62, 43, 40 and 35K)^{7,26,27}. The evidence that transcription and kinase activities are contained in TFIH is twofold. First, we found TFIH to fractionate in two different places on phosphocellulose, in the 0.5 M KCl and 1.0 M KCl washes. Highly purified TFIH derived from both fractions coelutes with both transcription and kinase activities. More importantly, the amounts of transcription and kinase activities (specific activity) recovered on the different chromatographic steps were indistinguishable. Second, monoclonal antibodies²⁷ produced against the recombinant 62K subunit of TFIH inhibited both transcription and kinase activities. This inhibition was specific as the antibodies could be neutralized by the addi-

tion of excess TFIH but not by an excess of other factors such as TFIIB. Thus, we conclude that both activities are contained in the same factor. A third line of evidence, though indirect, lies in the drastic kinase stimulatory effect exhibited by TFIIE. Because TFIIE is directly responsible for introducing TFIH to the preinitiation complex, and hence to RNA polymerase II, one would expect such a stimulatory capacity.

It is important to stress that phosphorylation of the CTD does not seem to be the only step during initiation of transcription that requires the hydrolysis of the β - γ bond of ATP. Initiation of transcription of different class II genes requires the specific hydrolysis of the β - γ bond of ATP³⁰. Because TFIH-mediated RNA polymerase II phosphorylation can use ATP or GTP as phosphate donor it is likely that another step, as yet undefined, specifically requires ATP hydrolysis during initiation of transcription.

We believe that TFIH (BTF2) is the human counterpart of the yeast general transcription factor b (ref. 18). Factor b contains an activity capable of phosphorylating the CTD of yeast RNA polymerase II (ref. 18). The 62K subunit of TFIH (BTF2) and the 75K subunit of factor b have recently been cloned (ref. 27, and O. Gileadi, R. G. Feaver and R. G. Kornberg, manuscript in preparation). Amino-acid sequence analysis revealed that p62 (from amino-acids 171-236) shares an overall similarity of 59% (41% identity and 18% similarity) with p75 of factor b (from amino-acids 233-302)²⁷. Although neither of the cloned polypeptides has a canonical kinase motif, both TFIH (BTF2) and factor b¹⁸ are capable of phosphorylating the CTD of RNA polymerase II. Unless renaturation studies prove fruitful, the identification of which subunits contain the catalytic kinase activity may await the cloning of all the subunits in each factor. TFIH (BTF2) may also be related to rat liver factor δ (ref. 31) because this factor seems to have a similar polypeptide composition, immunoreacts with 3c9 (ref. 27) and has been reported to contain a DNA-dependent ATPase activity³². The kinase activity contained in TFIH seems to be distinct from other reported mammalian kinases. Although the TFIH kinase activity requires DNA for optimal activity, this requirement is distinct from that observed with the DNA-dependent protein kinase¹⁵. The TFIH-associated kinase requires DNA sequences capable of directing formation of a transcription-competent complex; the DNA-dependent kinase is nonspecific in this respect. Although cdc2 can phosphorylate a CTD-containing peptide^{12,13}, TFIH does not contain cdc2-reacting material (unpublished observations) and neither use histone H1 as substrate. But we have found that highly purified preparations of MPF can phosphorylate RNA polymerase II resulting in inhibition of specific transcription (H.L., L.Z. and D.R., manuscript in preparation). It is possible that MPF and TFIH phosphorylate the CTD at different residues, and that phosphorylation at the 'MPF-specific site(s)' results in inhibition of transcription. □

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Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides

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Three-dimensional structures of complexes of the SH2 domain of the v-src oncogene product with two phosphotyrosyl peptides have been determined by X-ray crystallography at resolutions of 1.5 and 2.0 Å, respectively. A central antiparallel β -sheet in the structure is flanked by two α -helices, with peptide binding mediated by the sheet, intervening loops and one of the helices. The specific recognition of phosphotyrosine involves amino–aromatic interactions between lysine and arginine side chains and the ring system in addition to hydrogen-bonding interactions with the phosphate.

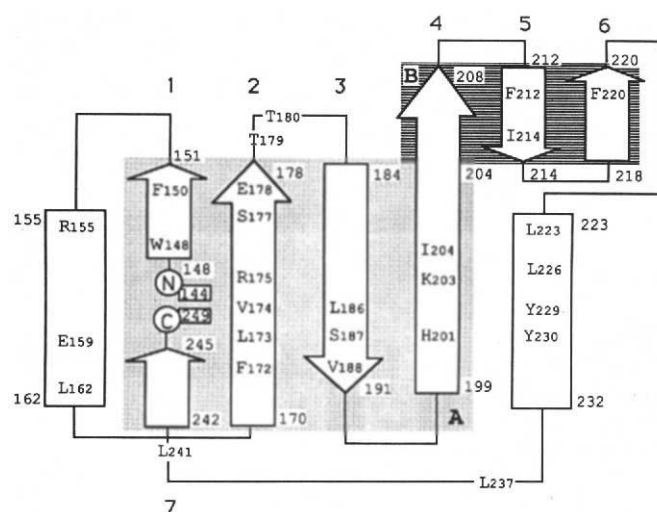
PROTEIN-TYROSINE kinases are enzymes that provide a central switching mechanism in cellular signal transduction pathways by catalysing the phosphorylation of tyrosine residues in specific proteins (for review, see ref. 1). Although the first tyrosine kinase was discovered as the protein product of the Rous sarcoma virus oncogene, p60^{v-src} (refs 2–4), the normal cellular forms of these enzymes occur either as transmembrane growth hormone receptors or as cytosolic non-receptor proteins associated with the inner surface of the plasma membrane. Activation of the transmembrane receptors results in the autophosphorylation of tyrosine residues in the cytoplasmic regions of the receptors⁵. Subsequent transmission of this ligand-induced signal is thought

to depend on the recognition of the phosphorylated tyrosines by distinctive domains containing approximately 100 amino-acid residues, known as SH2 (src homology-2) domains⁶. SH2 domains complement the action of the catalytic kinase activity by communicating the phosphorylation states of signal transduction proteins to elements of the signalling pathway, and are found in cytosolic non-receptor tyrosine kinases as well as in a number of other proteins that play key roles in signal transduction (for reviews, see ref. 7).

Signal transduction proteins that are not tyrosine kinases but which contain SH2 domains include, for example, phospholipase C- γ 1 (refs 8, 9), the p85 subunit of phosphatidyl-

FIG. 1 Schematic drawing of the secondary structure of the SH2 domain of the v-Src oncoprotein. Note that the sequence of the SH2 domain of c-Src is identical⁵². Arrows indicate β -strands and rectangles indicate α -helices. The numbers at the ends of the secondary structural elements are the sequence numbers of the boundary residues in Src SH2. Sheets A and B are indicated by shaded areas. Conserved or functionally important amino acids are shown.

METHODS. The gene segment encoding the SH2 domain of the v-Src tyrosine kinase (Schmidt–Ruppin A strain of the Rous sarcoma virus⁵³) between amino acids 144 to 249 was amplified using polymerase chain reaction. The 5' primer consisted of a *Nde*I restriction site followed by the sequence encoding the 5 N-terminal residues of the SH2 domain. The 3' primer consisted of the sequence encoding the five C-terminal residues of the SH2 domain, two termination codons (amber and ochre), followed by a *Bam*HI restriction site. The amplified fragment was cloned in the expression vector pET3a at the *Nde*I–*Bam*HI sites⁵⁴. The complete DNA sequence of the SH2 domain was confirmed by dideoxy sequencing. Cultures (2 litres) were routinely grown at 37 °C. After induction with 0.5 mM IPTG for 3 h, cells were harvested by centrifugation and resuspended in 100 ml of 20 mM HEPES, pH 7.5, 10 mM EDTA, 5 mM DTT, 5 μ g ml⁻¹ of leupeptin (Boehringer, Mannheim, FRG), and 1% (v/v) aprotinin (Sigma). Cells were lysed using sonication and lysates were centrifuged for 20 min at 15,000 r.p.m. at 4 °C. Supernatants were directly loaded onto a 20-ml S-Sepharose Fast Flow column (Pharmacia) pre-equilibrated in 20 mM HEPES, pH 8, 5 mM EDTA and 5 mM DTT. The protein was then eluted using a NaCl gradient (0 to 0.5 M), in the same buffer. A single peak containing the protein was eluted at 0.35 M salt. Purity was above 99% as estimated using SDS–PAGE electrophoresis. N-terminal sequencing and mass spectroscopy showed that



the protein was unmodified during expression and purification. The measured mass is 12,298 $\pm$ 2, compared with 12,300 calculated from the v-Src sequence and including an N-terminal methionine.

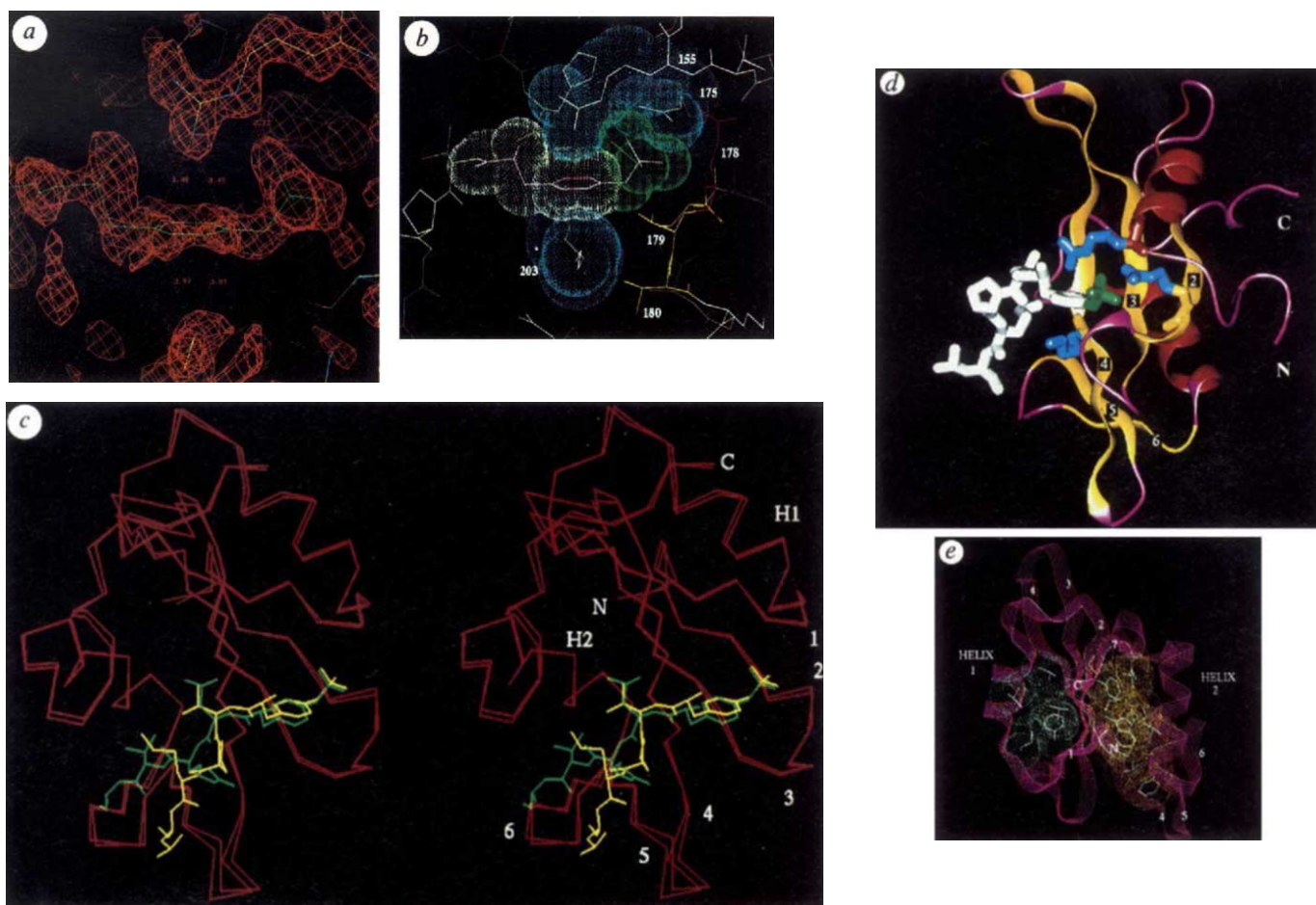


FIG. 2 *a*, Refined atomic model for the peptide A complex at the phosphotyrosine binding site, and electron density from a simulated annealing omit map. Arg 155, Arg 175, Lys 203 and the entire peptide were deleted from the final model and simulated annealing refinement was used to remove bias due to these residues from the calculated phases³¹. The electron density map was calculated using $(|F_o| - |F_c|)$ coefficients at 1.5 Å resolution, where F_o is the observed structure factor and F_c is that calculated from the partial model. Although noisier than maps calculated using the entire model, this unbiased map reveals all features of the phosphotyrosine recognition. The phosphotyrosine sidechain is in green and the sidechain of Arg 155 is above it. *b*, The atomic model in *a* is shown with dot surfaces representing van der Waals radii. White dots indicate the aromatic ring, with the phosphate ring in green. The blue dots indicate the positively charged residues. Residues of the phosphate binding loop are shown in yellow (Ser and Thr) and red (Glu). Note the close interaction between the terminal nitrogen of Arg 155 and the tyrosine ring. Lys 203 and Arg 175 can be seen below the ring and behind the phosphate group, respectively. *c*, Stereo-drawing of the SH2/peptide A and SH2/peptide B complexes. The C α backbone of the SH2 domain is indicated in red for both structures, which were superimposed by using protein atoms alone. All atoms of peptides A and B are shown in yellow and green, respectively. *d*, Schematic ribbon drawing of the SH2 domain of the peptide A complex. The peptide (in white) and the three charged residues at the binding site (in blue) are shown explicitly. The phosphate group is shown in green. Arg 155 is above the phosphotyrosine ring, Arg 175 is to the right of the phosphate group and Lys 203 is below the ring. α helices are shown in red, β strand in yellow and non-regular structure in pink. Strands 2 to 6 are indicated by numbers, and strands 1 and 7 are not indicated explicitly. *e*, Hydrophobic cores of the SH2 domain. Yellow dots indicate the van der Waals surfaces of the sidechains that constitute the hydrophobic core between helix 2 and sheets A and B (residues 148, 172, 174, 186, 188, 204, 212, 214, 223, 226, 227, 237, 241). Green dots indicate the same for the hydrophobic cluster between helix 1 and sheet A (residues 150, 153, 161, 162, 173). The side chain of

Trp 148 is clearly visible, with that of Phe 172 directly above it.

METHODS. The atomic model obtained using the MIR phases was refined against 3.0 Å native X-ray data using the program X-PLOR⁵⁵. Repeated model building using 'O'⁴⁹ and least squares refinement with simulated annealing using X-PLOR were carried out⁵⁶. The resolution of the native data was extended to 1.5 Å, and 58 well resolved solvent molecules (interpreted as water) were included. The current model has all SH2 residues from Gln 145 to Thr 247 and the 5 residue peptide built in. Stereochemical parameters for the phosphate group were based on the crystal structure of phenyl phosphate⁵⁹. The *R* factor is 20.7% at 1.5 Å for 14,798 reflections between 6 Å and 1.5 Å with $|F| > 2\sigma(F)$ (this includes 98% of the measured data). The rms deviation from ideal geometry is 0.014 Å for bond lengths and 3.0° for bond angles, with good stereochemistry for the backbone torsion angles and tightly restrained temperature factors. Although the density is weak for a few side chains, the backbone density is generally strong throughout the structure. The loop connecting strands 3 and 4 is in poor density, and appears to be disordered. Crystals with peptide B are in space group $P4_1$ ($a=b=47.9$ Å, $c=53.4$ Å), with one molecule in the asymmetric unit. The X-ray dataset for the peptide B complex is 91% complete to 2.0 Å, with average $F/\sigma(F)$ of 6.5 in the shell from 2.25 Å to 2.0 Å, and an overall R_{symm} of 6.7%. The structure of the SH2/peptide B complex was determined using molecular replacement, by the method of Brünger⁵⁷, using the structure of the peptide A complex (without peptide or water molecules) as the search model. The rotation and translation function solutions were unambiguous and led to the correct enantiomorphic choice of space group as $P4_1$. The density for the peptide B was clearly defined in difference maps and a complete model for it was built and 30 solvent molecules were included in least-squares refinement. The *R*-factor is 18.9%, including 6,514 reflections between 6 Å and 2 Å resolution with $F > 2\sigma(F)$ (96% of the measured data). The r.m.s. deviation from ideal geometry is 0.012 Å for bond lengths and 2.8° for bond angles. The r.m.s. deviation between the two peptide/SH2 complexes is 0.36 Å for the C α atoms, excluding residues in the disordered loop between strands 3 and 4.

inositol-3-OH kinase¹⁰, *ras* GTPase-activating protein (GAP)¹¹, and protein-tyrosine phosphatases¹². In each of these proteins the sequence of the SH2 domain is highly conserved and is thought to be important in either localizing the protein to tyrosine-phosphorylated proteins or controlling its activity. In addition, many of these SH2-containing proteins also contain another class of sequence module known as the SH3 domain, the functions of which are less clearly understood⁷.

The functional importance of the SH2 domain was made clear from studies on the oncogene product p47^{gag-crk}, which causes cellular transformation and increased levels of tyrosine phosphorylation but does not contain a kinase domain¹³. p47^{gag-crk} does, however, contain SH2 and SH3 domains. The SH2 domain is solely responsible for association with tyrosine phosphorylated proteins and, with SH3, is required for cellular transformation^{14,15}. Studies on a number of isolated SH2 domains have shown that they can associate independently with tyrosine-phosphorylated proteins^{16,17}. The most extensive mutagenesis studies have been on the SH2 domains of the viral and cellular *src* tyrosine kinase (for example, see refs 18–20). It has been shown that certain alterations in the SH2 domain can activate the transforming potential of p60^{c-src}. A simple auto-inhibitory model proposes that the SH2 domain of p60^{c-src} binds to a phosphorylated tyrosine in the C-terminal region of the enzyme (Tyr 527), thereby blocking the active site of the kinase domain²¹. Other mutations within the SH2 domain of p60^{v-src} and p60^{c-src} also result in transformation-defective or host-cell-dependent

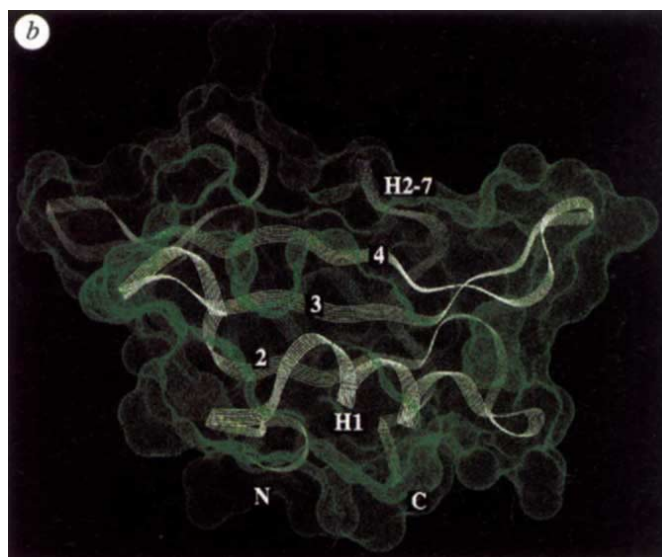
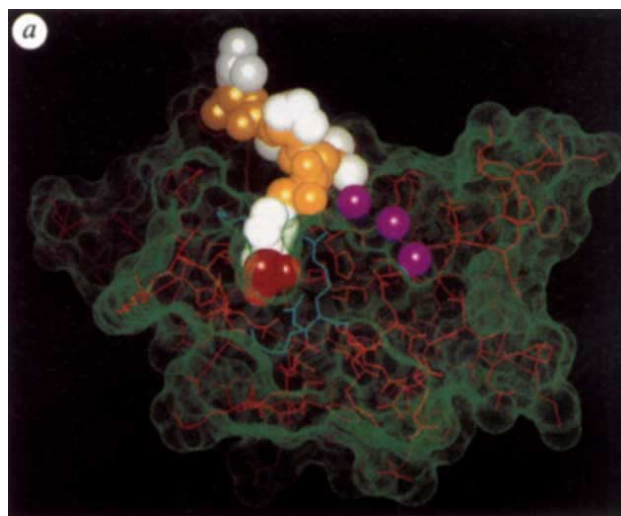
transformation phenotypes, and these have highlighted the importance of interactions between SH2 and (as yet unknown) cell-specific factors^{22,23}.

A more complete understanding of the mechanism of SH2 action requires detailed structural information to complement the genetic and biochemical information currently available. We present here the first crystal structure of an SH2 domain, that of the *v-src* tyrosine kinase. The structures reported are for the complex of the SH2 domain with two different pentapeptides containing phosphotyrosine, determined at 1.5 Å and 2.0 Å resolution, and they complement structural information obtained by NMR in solution for the peptide-free form of *c-abl* SH2 (refs 24, 25). These structures provide the first view of the mechanism by which phosphorylated tyrosine residues are recognized by SH2 domains and will facilitate new protein engineering experiments and the design of SH2-specific inhibitors. The structures make possible the application of computer modelling techniques to the family of SH2-containing proteins, which was not possible previously because of the lack of sequence similarity to any protein of known three-dimensional structure.

X-ray analysis of peptide-SH2 complexes

The boundaries of the SH2 domain of the *v-src* tyrosine kinase were defined previously by sequence alignment⁷. A segment of the *v-src* gene encoding 106 amino acids, spanning residues 144–249, was expressed in *Escherichia coli* (Fig. 1). The purified

FIG. 3 Solvent accessible surface area⁵⁸ of the SH2 domain. The green dots indicate the surface traced out by a sphere of 1.4 Å radius rolling over the protein molecule (with all waters and the peptide removed from the model). *a*, Non-hydrogen atoms of the SH2 domain are shown in red, with Arg 155, Arg 175 and Lys 203 in blue. Peptide A is shown as a space filling model, with yellow and grey spheres indicating the backbone and sidechain atoms, respectively. The phosphate group is in red. Purple spheres indicate a shallow groove on the surface that might provide binding sites for longer peptides. *b*, The backbone structure of the SH2 domain is shown as a white ribbon underlying the same surface as in *a*. The numbers indicate some of the strands. Helix 1 is indicated as H1. Helix 2 is not clearly visible. H2–7 indicates the loop connecting helix 2 with strand 7.



form of this Src-SH2 domain is extremely soluble ($>300 \text{ mg ml}^{-1}$ in aqueous buffer) and is obtained in high yield (50 mg per litre of culture). The identity of the purified protein was confirmed by N-terminal amino-acid sequencing and by high-resolution mass spectroscopy (Fig. 1).

Peptides containing phosphorylated tyrosine residues and with sequences corresponding to 5–10-residue regions of the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors and the C-terminal region of *c-src* protein have been shown to bind to SH2 domains^{21,26,27}. Several peptides with sequences related to these were screened for crystallization with Src SH2 but only two yielded usable crystals. These consisted of residues 751–755 of the β -subunit of the human PDGF receptor (Tyr-Val-Pro-Met-Leu, peptide A)²⁷ and residues 1,174–1,178 of the human EGF receptor (Tyr-Leu-Arg-Val-Ala, peptide B)²⁶, each phosphorylated on tyrosine. Addition of phosphotyrosine alone to Src SH2 did not lead to crystallization. The relative affinity of the peptides for the Src SH2 domain was assayed by competitive ELISA²⁸. In these experiments, immobilized EGF receptors from unstimulated A431 cells were autophosphorylated *in vitro* and incubated with pre-mixed solutions of Src SH2 (expressed for these experiments as a glutathione *S*-transferase conjugate) and serially diluted phosphopeptides (concentrations from 10^{-2} to 10^{-8} M). Both peptides have relatively low affinity for Src SH2, with approximately 1 mM peptide required for 50% inhibition of SH2 binding to the EGF receptor (data not shown). Nevertheless, it is possible to inhibit Src SH2 binding to the target protein with peptide concentrations similar to those used for crystallization (20 mM), indicating that these peptides do satisfy minimal structural requirements for functional binding. This affinity is much lower than that reported for specific SH2-protein phosphotyrosine

interactions^{29,30}; consequently the structures described here do not provide direct information on the determinants of sequence-specific binding.

Addition of either peptide A or B to Src SH2 has a dramatic effect on crystallization properties. In the absence of phosphorylated peptide, the protein crystallizes from polyethylene glycol solutions and phosphate buffer to yield orthorhombic crystals (space group $P2_12_12_1$; $a = 110.9 \text{ \AA}$, $b = 86.2 \text{ \AA}$, $c = 58.9 \text{ \AA}$) with four molecules in the asymmetric unit. Although these crystals diffract to high resolution, structure determination was not possible owing to difficulties in obtaining large single crystals, as well as a high degree of non-isomorphism from crystal to crystal. In the absence of phosphate or peptide, large hexagonal crystals are readily obtained that are not suitable for structure determination because of disorder in the crystal packing along certain lattice directions. A striking change in crystallization behaviour was observed upon addition of stoichiometric amounts of peptides A or B to Src SH2. Crystallization is extremely rapid (within hours) and large single crystals are obtained reproducibly, with strong diffraction observed to $1.5 \text{ \AA}$ resolution in both cases, suggesting that a stable complex is formed (Table 1). Co-crystals of Src SH2 with peptide A are orthorhombic (space group $P2_12_12_1$, $a = 46.3 \text{ \AA}$, $b = 57.5 \text{ \AA}$, $c = 39.3 \text{ \AA}$), whereas those obtained with peptide B are tetragonal (space group $P4_1$, $a = b = 47.9 \text{ \AA}$, $c = 53.4 \text{ \AA}$). Both crystal forms have only one SH2-peptide complex in the asymmetric unit, in contrast to the four molecules observed with the isolated protein. The packing of the protein molecules in the crystal lattice is completely different in the two crystal forms of the peptide complexes. This is fortunate, because structural details that are common to both crystal forms are unlikely to be distorted by crystal-packing forces, a point that is of particular importance in analysing

TABLE 1 Statistics for multiple isomorphous replacement

	Native peptide A	Native peptide B	Uranyl acetate 10 mM	Uranyl acetate 1 mM	Iridium chloride 100 mM	Iridium chloride 10 mM	EMP 1 mM
Resolution	1.5 Å	2.5 Å	2.5 Å	2.5 Å	2.5 Å	2.5 Å	2.5 Å
$R_{\text{symm}}(I)$	3.3%	3.5%	5–6%	5–6%	5–6%	5–6%	5–6%
Fractional isomorphous difference		6.5%	39%	12%	18%	10%	15%
No. of sites			6	5	5	4	4
Phasing power			2.2	0.9	1.6	1.6	1.4

All derivative datasets were obtained using glutaraldehyde crosslinked crystals of the SH2/peptide A complex. A native dataset collected using a crosslinked crystal (second column) was used for the calculation of MIR phases. However, crystallographic refinement of the complete model was carried out with reference to the $1.5 \text{ \AA}$ resolution dataset obtained using a non-crosslinked crystal (first column). $R_{\text{symm}}(I)$: agreement value between the intensities of symmetry related reflections. Fractional isomorphous difference: Average change in structure factor between native and derivative data, calculated with reference to the crosslinked native dataset for all derivatives. The isomorphous difference between the unmodified and crosslinked native crystals is also shown. **Preparation of the peptides:** Two pentapeptides (A: YVPML; B: YLRVA) phosphorylated on the tyrosine were used. Solid phase peptide synthesis was used on a DuPont RaMPS multiple peptide synthesis system. C-terminal amino acids attached to Wang resin were obtained from DuPont/NEN. All amino acids were coupled as their N-Fmoc pentafluorophenyl esters (Advanced Chemtech), except for N-Boc-O-dibenzylphosphono-L-tyrosine (Peninsula) which was coupled as its butanol ester, and double coupling was employed. Cleavage of peptide from resin was achieved using a mixture of trifluoroacetic acid–EDT–aqueous phenol for 4 h. Peptides were purified by reverse-phase HPLC on a Merck C-18 column using a 28% to 43% gradient of 0.1% trifluoroacetic acid in acetonitrile over 20 min. All peptides were characterized by ^1H and ^{31}P NMR and high-resolution mass spectroscopy, and were greater than 95% pure. **Crystallization:** The purified protein was concentrated to 250 mg ml^{-1} (20 mM) using ultrafiltration. The peptides were dissolved in water to 20 mM, and mixed with equal volumes of protein solution. Crystals were grown at room temperature using vapour equilibration against a solution of 10% polyethylene glycol (average $M_r = 4,000$) in 100 mM HEPES buffer at pH 8, starting with mixtures of equal volumes of protein/peptide and polyethylene glycol solutions. **Data collection and structure determination.** SH2-peptide A crystals are in space group $P2_12_12_1$ ($a = 46.3 \text{ \AA}$, $b = 57.5 \text{ \AA}$, $c = 39.3 \text{ \AA}$), with one molecule in the asymmetric unit. X-ray diffraction data were measured using a Rigaku R-Axis IIC imaging plate area detector, mounted on a Rigaku RU200 rotating anode X-ray generator, using one crystal for each dataset. The native dataset is 90% complete to $1.5 \text{ \AA}$, with average $F/\sigma(F)$ of 7.2 in the resolution shell from $2.0 \text{ \AA}$ to $1.5 \text{ \AA}$. The search for suitable heavy-atom derivatives was aided by crosslinking the crystals with glutaraldehyde^{44,45}. Crystals were stabilized in 30% polyethylene glycol, 100 mM HEPES, pH 8, and then transferred to 0.5% glutaraldehyde in stabilizing solution for 30 min at room temperature, followed by transfer to 0.5% *n*-butylamine in stabilizing solution for 20 min. Including the last step led to more reproducible results, presumably as a result of the capping of partially reacted glutaraldehyde molecules. All derivative datasets were referred to the crosslinked native dataset. Uranium acetate, sodium hexachloroiridate and ethyl mercury phosphate (EMP) gave useful derivatives at the concentrations indicated. Anomalous dispersion measurements were included for all derivatives. Heavy atom parameters were refined and initial phases were calculated using the program HEAVY⁴⁶. The MIR phases were further improved by solvent flattening using a program written by W. Kabsch (COMBINE)⁴⁷, and an electron density map was calculated at $3 \text{ \AA}$ resolution. A partial model of polyaniline chain was built, using the program 'O' and a database of protein structures^{48,49}. Phases from the partial atomic model were combined with MIR and solvent-flattened phases, including MIR data to $2.5 \text{ \AA}$. The interpretation of the MIR map was aided by information from NMR regarding the secondary structure of the *c-abl* SH2 domain²⁴, and a complete atomic model was readily built. The alignment of the amino acid sequence in the electron density map is consistent with mercury atoms (from EMP) binding to all three cysteines (Cys 185, Cys 238 and Cys 245) and one histidine (His 233).

peptide interactions that occur at the surface of the SH2 molecule.

The structure of the peptide A complex was determined by multiple isomorphous replacement (MIR) (Table 1). The interpretation of the MIR electron density map was aided greatly by knowledge of the likely positions of secondary structural motifs, obtained from NMR experiments on the *c-abl* SH2 domain, in solution and without peptide²⁴. In particular, definite knowledge about the antiparallel nature of the β -sheet at the core of the structure and the lengths of certain loop connections between strands and helices allowed a model to be built through a few regions of ambiguously connected electron density. The X-ray structure determination from the MIR-derived electron density map involved no other direct input from the NMR-based determination of the three-dimensional structure of the *c-abl* SH2 domain, which proceeded independently²⁵.

An accurate atomic model has been refined for the peptide A complex at 1.5 Å resolution (Fig. 2). The structure of the peptide B complex was readily determined by molecular replacement, and it was apparent that the mode of phosphotyrosine binding is identical (Fig. 2c). The discussion that follows is therefore limited to the peptide A complex unless stated otherwise.

Topology of the secondary structure

The core element of the structure is an antiparallel β -sheet that is sandwiched between two α -helices (Figs 1, 2). The N terminus

of the domain leads to a three-residue irregular β -strand (strand 1) that runs parallel to and is hydrogen-bonded with the first strand (strand 2) of a five-stranded antiparallel network. Strand 1 is critical to the integrity of the structure as it maintains the absolutely conserved side chain of Trp 148 in a position where it packs against other conserved side chains to form the hydrophobic core of the structure (Fig. 2e). Strand 1 leads into helix 1, at the N-terminal end of which is Arg 155, which recognizes both the phosphate group and the tyrosine ring. Strand 2 contains a highly conserved sequence motif: Phe-Leu-Val-Arg. Phenylalanine 172 and Val 174 of this motif pack against Trp 148 and the remainder of the hydrophobic core, whereas Leu 173 is involved in maintaining the orientation of helix 1 (Fig. 2e). Arginine 175 is completely buried in the structure and forms an ion pair with the phosphate group of the peptide. The C-terminal end of strand 2 and the loop following it provide most of the other binding interactions with the phosphate. This loop, connecting strands 2 and 3, will be referred to as the 'phosphate-binding loop'. The importance of strand 2 and this loop has been identified by mutagenesis studies¹⁷.

The central β -sheet can be subdivided into two connected β -sheets. Strands 2, 3 and the N-terminal part of strand 4 constitute the base of sheet A, with the irregular strands 1 and 7 also providing hydrogen-bonding interactions. The C-terminal part of strand 4 along with strands 5 and 6 constitutes the much smaller sheet B, which curves away from the base (Fig. 2d) and provides a cap to the hydrophobic core (Fig. 2e). The C-terminal

TABLE 2 Structural alignment of the sequences of SH2 domains

	Strand1		Helix1		Strand2		Strand3		Strand4+4'		Strand5		Strand6		Helix2		strand7		Z score								
1	B	A	B		BBBA AA AA	B B	B A AB	B	B	B	B	B	B	B	B	B	B	B									
v-src	WYF	GKIT	RRESERLL	LNPENR	GT	FLVRESE	TTK	GA	YCLSVSDF	DNAA	GLN	VKHYKI	RKL	DS	GG	FYI	TSR	TQF	SS	LQQLVATYSN	HADG	LCHR	L	TNV	C	31.3	
yes	WYF	GRMG	RKDAERLL	LNPQNR	GI	FLVRESE	TTK	GA	YSLSIROW	DEIR	GDN	VKHYKI	RKL	DN	GG	YYI	TTR	AQF	DT	LQKLVKHYTE	HADG	LCHK	L	TTV	C	29.2	
hck	WYF	KGIS	RKDAERQL	LAPGNR	GS	FLVRESE	TTK	GS	YSLSVROD	DPQ	GGT	VKHYKI	RTL	DN	GG	FYI	SPR	STF	ST	LQELVDHYKK	GNDG	LQK	L	SVP	C	27.3	
fgr	WYF	KGIG	RKDAERQL	LSPGNQ	GA	FLVRESE	TTK	GA	YSLSIROW	DQTR	GDH	VKHYKI	RKL	DM	GG	YYI	TTR	VQF	NS	VQELVQHYME	VNDG	LCNL	L	IAP	C	26.3	
fyn	WYF	KGIG	RKDAERQL	LSPGNPR	GT	FLVRESE	TTK	GA	YSLSIROW	DDMK	GDH	VKHYKI	RKL	DN	GG	YYI	TTR	AQF	ET	LQQLVQHYSE	RAAG	LCCR	L	VVP	C	25.6	
ick	WYF	KNLS	RKDAERQL	LAPGNH	GS	FLVRESE	STA	GS	FSLSVROD	DQNG	GEV	VKHYKI	RNL	DN	GG	FYI	SPR	ITF	PG	LQDLVRYHYN	ASDG	LCTK	L	SRP	C	25.3	
lyn	WYF	KDIT	RKDAERQL	LAPGNSA	GA	FLVRESE	TLK	GS	FSLSVROD	DPVH	GDV	IKHYKI	RSL	DN	GG	YYI	SPR	ITF	PC	LQALVQHYSK	KDGG	LQK	L	TLR	C	24.0	
Src	WYV	GYMS	RQRAESLL	KQDKE	GC	FVVRKSS	TK	GL	YTLISLHTK	VPQSH		VKHYKI	RQN	ARCE		YYL	SEX	H	CCET	IPDLINHYRH	NSGG	LACR	L	KSS	PC	14.5	
abl	WYH	GPVS	RNAAEYLL	SSGIN	GS	FLVRESE	SSP	GO	RSISIRYE	G	R	VYHYRI	NTAD		GK	LYV	SSE	SRF	NT	LAEIVHHIST	VADG		L	ITT	L	14.3	
arg	WYH	GPVS	RSAAEYLL	SSLIN	GS	FLVRESE	SSP	GO	LSISIRYE	G	R	VYHYRI	NTAD		CK	VYV	TAE	SRF	ST	LAEIVHHIST	VADG		L	VTT	L	13.7	
Dabl	WYH	GPIS	RNAAEYLL	SSGIN	GS	FLVRESE	SSP	GO	RSISIRYE	G	R	VYHYRI	SEDP		GK	VYV	TQE	AKF	NT	LAEIVHHIS	VPHECH		L	ITP	L	13.4	
nck	WYI	GKVT	RHQAEML	NERGHE	GD	FLVRESE	SSPD	GS	FSLSVKAO	G	K	NKHFV	OLKE			VYC	GQRK	F	ST	MEELVEHYKK	AP	IFTSECEK	L	YLV	K	11.2	
gapC	WYH	GKIS	RQAEYLL	MTV	GGACS	FLVRPSD	NTP	GD	YSLYFRYS	EN		IQRFKI	CPTPNQ			FMH	GGR	YY	NS	IGDIDHYRK	EQ	IVEGYI	L	KEP	V	10.7	
csk	WYH	KEIT	RQAEYLL	YPPET	GL	FLVRESE	NYP	GD	YTLVCSVE	G	K	VEHYRI	MYHASK			LSI	DEE	VYH	EN	IGDIDHYRT	DAGC	LCTR	L	IKP	K	9.8	
plcg2N	WYH	KKVES	RTSAEKLL	QEQCAET	GAKDGT	FLVRESE	TFPND	YTSLFWRS	G	R	VQHCRI	RSTHE			GGVKK	YYL	TNET	F	NS	LYALQHYRE	AHLRC	AFFE	L	YLV	K	9.3	
gapN	WYH	KLD	RTIAERLL	ROAGKS	GS	FLVRESE	RHP	GS	FVLSVFSQ	TNV		VNHFR	IAMC		GD	YYI	GGR	F	SS	LSGLGYSSH	VSC	LLAGE	L	LYP	V	9.2	
plcg1N	WYH	KLAGAGRG	RHIAERLL	TEYCIET	GAPOGS	FLVRESE	TFV	GD	YTSLFWRN	G	K	VQHCRI	HSRQDA		GTPK	FPL	TDLIV	F	DS	LYDLITHYQQ	VP	LRCNEFEM	L	SEP	V	9.2	
p85a	WYH	GDIS	REEVNEKL	RTDAD	GT	FLVRDAS	TKMH	GD	YTSLFRK	GGNN	KLIK	FHRD			GKY	GFSOP	LYT	NS		NVELINHYRN	ES	LAQYNP	L	DVK	L	8.2	
ptp1c	WYH	GHSOG	QAEYLL	QAK	GEPWT	FLVRESL	SQP	GD	FVLSVLSQ	QFKA	G(6	VTHIR	NCE		GGR	Y	TVGGLET	F	DS	LDLVEHFKK	TC	TEASG	A	FVY	L	7.8	
crk	WYH	GRLS	RGDAYSLL	QGGH	GT	FLVRDSG	SIP	GD	FVLSVSES	SR		VSHYIV	NSLGPAGRRAGE	(18)					F	DS	LPSELLEYKI	HY	LDITT	L	IEP	V	7.8
fer	WYH	GAIP	RIEAQELL	KKQ	GD	FLVRSH	GKP	GE	YVLSVYSQ	GQR	RHFII	OYDNNYFE	GTC						F	SN	IPQLIDHHTY	TQ	VITKSG	V	VLL	N	7.7
p85b	WYH	GDIS	REEVNEKL	RTDAD	GT	FLVRDAS	SKIQ	GE	YTSLFRK	G	NN	KLIK	FHRD		GNY		GFSEP	LYT	CS	VVDLITHYRN	ES	LAQYNAK	L	OTR	L	6.8	
ptp1cN	WYH	RDLSG	LDIAETLL	KGRGVH	GS	FLVRPSR	KNQ	GD	FSLSVRV	GDQ	VTHIR	ONS		GD	FY	DLYGCK	F	AT		LTSLVEYITO	QGV	LQORDGT	L	TH	L	5.6	
plcg1C	WYH	ASLT	RAQAEHML	MVPRD	GA	FLVRKR	EPNS	YALSFRAE	G	K	IKHCRV	QOE		GQT		VML	GNSE	F	DS	LVDLSYERK	HP	LYRMM	L	RYP	I	5.6	
plcg2C	WYI	DRLS	RGEAEML	NRIPO	GA	FLIRKRE	GTDS	YAITFRAR	G	K	VKHCRI	NBD		GRH		FVL	GTSAY	F	ES	LVDLSYERK	H	ALYRMM	L	RYP	V	5.4	
tensin	WYH	PDIS	RQDAIALL	KOREP	GA	FLVROSH	SFR	GA	YGLAMKVA	SPPP	(15)	VHRLFI	TSRP		GVK	L	KCCPNSPR	F	GC	LSALVYQHSI	MP	LALPCK	L	VIF	D	5.7	
fps	WYH	GAIP	RSEVQELL	KCS	GD	FLVRESQ	GKQ	E	YVLSVWLD	GQP	RHFII	QANDLYRE	GDC						F	PT	IPLLIDHL	LQSQOP	L	TRK	S	4.2	
p85c	WYH	GKIN	RTQAEML	SGKRD	GT	FLVRESS	QR	GC	YACSVVD	GDT	KHCVI	YRTAT		G	FCF	AEPYNL	Y	GS		LKELVHYOH	AS	LQVHND	L	TVT	L	4.0	
vav	WYA	GPME	RGAESILL	ANRSD	GT	FLVRQV	KDAA	E	FAISIKYN	VE		VKHYTK	IMTAE		GL	YRI	TEKRA	F	RG	LKELVEFYQD	NS	LKDCFKS	L	DTT	L	2.0	
p85aC	WNV	GSSN	RKKAENLL	RGKRD	GT	FLVRESS	KQ	GC	YACSVVD	GE	VKHCVI	NKTAT		G	YCF	AEPYNL	Y	SS		LKELVHYOH	TS	LQVHNS	L	NVT	L	1.7	

Twenty six of the sequences of SH2 domains listed by Koch *et al.*⁷, as well as four additional ones (d-Src⁵⁰, Csk⁵¹ and two from a soluble protein tyrosine phosphatase, PTP1C¹²) are ranked according to their potential structural similarity with Src SH2. The Z-score, from the profile method of alignment, is given in the last column⁴². A database containing 252 sequences in addition to the SH2 sequences was used to define the Z-scores. Sequences unrelated to SH2 are found to have Z-scores of 3.8 or lower. Two SH2 sequences (vav and p85aC) have Z-scores below this cutoff; nevertheless, they are clearly SH2 domains. The profile method was only used to rank the sequences and the particular alignment shown was done by manual inspection. Line 1 defines the residues in Src SH2 that are part of the phosphotyrosine binding site (A), and that are buried and in a hydrophobic environment (B). Boxes delineate the secondary structure elements, and are extended when possible to the sequences of other SH2 domains. A shaded area is shown when this extension is ambiguous. Although strand 4 is continuous in the structure, it is shown as two strands (4 and 4') in the table to emphasize its participation in sheet A (strand 4) and sheet B (4').

helix (helix 2) is an essential element of the hydrophobic core and packs against sheet A on the side opposite from helix 1 (Fig. 2e). Finally, an interesting feature of the structure is that the last strand brings the N- and C-termini into close proximity, on the side of the domain opposite to the peptide-binding surface. This emphasizes the modular nature of the SH2 domain and implies that it can be inserted as a unit into various regions of other protein structures without diminishing its ability to fold into a functional form.

The two α -helices run parallel to each other and are in fairly symmetrical positions on either side of sheet A. However, helix 2 is positioned approximately 1 Å more distant from the sheet than helix 1. The increased distance is maintained by 13 hydrophobic residues that pack between the helix and both sheets, and form the hydrophobic core. These include many residues that are strongly conserved across SH2 domains (Fig. 2e). In addition, 5 residues on the other side of the sheet pack to form a smaller hydrophobic cluster between helix 1 and sheet A (Fig. 2e).

General features of peptide binding

The overall shape of the SH2 domain resembles a somewhat flattened hemisphere, the cross-sectional face of which provides the platform for peptide binding (Fig. 3). This surface is roughly perpendicular to the plane of sheet A, and is formed by the juxtapositioning of the exposed edge of sheet A (strand 4), the edge of helix 1, the phosphate-binding loop, the loop between strands 5–6 and the loop following the C-terminal end of helix 2 (Fig. 3). The binding surface is relatively flat, with no evidence for a deep crevice. The phosphotyrosine residue binds in a small cleft formed by Arg 155, the phosphate-binding loop, and two side chains from strand 4 (Lys 203 and His 201). Shallow grooves formed by other side chains are evident on the binding surface on either side of the phosphotyrosine. Peptides A and B both have the phosphotyrosine residue at the N terminus, and both are bound roughly in the same groove (Fig. 2). Potential binding sites for residues upstream of the phosphotyrosine are indicated in Fig. 3.

Of the five residues in each peptide, the side chains at positions 1 (the phosphotyrosine), 2 (Val in A and Leu in B) and 4 (Met and Val) make contacts with the SH2 domain. The conformation of the phosphotyrosine residue is similar in both complexes, despite completely different crystal-packing environments, and is described in the next section. In contrast to the multiple protein-peptide interactions observed at the phosphotyrosine binding site, the interactions at positions 2 and 4 are quite loose, and these structures do not clearly define the basis for sequence-specific recognition. The Val and Leu at position 2 pack against the hydrophobic surfaces of the side chains of Tyr 202 and Lys 200. The carbonyl oxygen of His 201 is hydrogen-bonded to the amide nitrogen at position 2 of both peptides. The side

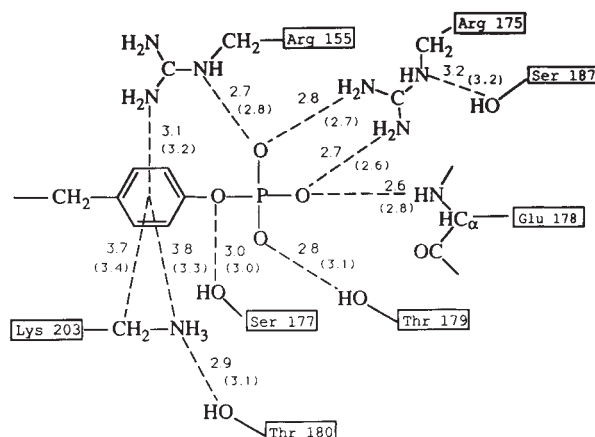
chains at position 4 (Val and Met) differ in length. Nevertheless, the backbones of the peptides diverge in order to bring the terminal atoms of the valine into the same position as those of the methionine, at a site of mixed polarity formed by the side chains of Ile 114, Tyr 202 and the methyl group of Thr 215. The relatively large average deviation of $C\alpha$ positions for the first 4 residues (1.7 Å) is due to distortions in the backbone that result in positioning the terminal atoms of Val 4 (in A) and Met 4 (in B) at the same site. Residues at the fifth position do not interact directly with the protein, and are in quite different conformations in the two peptides.

Specific recognition of the phosphotyrosine

Three positively charged residues (Arg 155, Arg 175 and Lys 203) are in close proximity to the phosphotyrosine binding site. Arginine 175 (of the conserved Phe-Leu-Val-Arg sequence in strand 2) forms an ion pair with the phosphate group, with specific hydrogen-bonding interactions between the two terminal nitrogens and two of the phosphate oxygens (Fig. 4). Arginine is the only residue capable of forming this dual interaction, and mutation of this residue to lysine in the *c-abl* SH2 domain leads to a loss of binding¹⁷.

A remarkable feature of the structure is that the two other charged residues at the active site interact directly with the phosphotyrosine ring, which has no aromatic or hydrophobic side chains in its vicinity (Fig. 2). Arg 155 is particularly important, as it is involved in simultaneous recognition of the phosphate group and the aromatic ring. A strong hydrogen bond is formed between the non-terminal nitrogen ($N\epsilon$) and a phosphate oxygen ($O-N$ distance of 2.7 Å). Simultaneously, one of the terminal nitrogens is 3.1 Å above the centre of the aromatic ring of the phosphotyrosine, in close contact with all six carbon atoms. This is an optimally constructed amino-aromatic interaction (see below). The side chain of Lys 203 crosses underneath the aromatic ring (Figs 2, 3), and is held in place by a hydrogen bond between the terminal amino group and the hydroxyl group of Thr 180. Lysine 203 makes no direct interactions with the phosphate group. Instead, the carbon atoms of the side chain appear to form a hydrophobic platform for the ring, with C-C distances of approximately 3.7 Å between the lysine and the ring. The terminal amino-group is positioned 3.8 Å below the edge of the ring, with the formation of a close to optimal amino-aromatic interaction. These interactions were first recognized in the MIR map at 3 Å resolution, and they persisted throughout the extension of refinement to high resolution. They have been unambiguously confirmed in the final model by omitting the peptide and all three charged residues from the model and carrying out simulated annealing refinement. Difference electron density maps calculated using the resulting structure are not biased by these residues³¹, and these maps recapitulate all features of the refined model (Fig. 2).

FIG. 4 Schematic drawing of the phosphotyrosine binding site. Dashed lines indicate interactions with the tyrosine ring and hydrogen bonds. Distances between non-hydrogen atoms (and with the centre of the ring) are shown for the peptide A complex, with the corresponding distances for the peptide B complex in parentheses.



Amino–aromatic interactions such as this were first seen in structures of haemoglobin–drug complexes³², and analysed by Burley and Petsko, who surveyed a number of X-ray structures and demonstrated a marked tendency for amino groups to be found above and below the aromatic ring and close to the centre³³. For example, in bovine trypsin inhibitor a tyrosine ring that is sandwiched between two amino groups has been observed by X-ray and neutron diffraction, as well as by NMR in solution^{34,35}. More recently, the crystal structure of acetylcholine esterase reveals that the positively charged amine of the substrate is stabilized by the aromatic ring of a tryptophan side chain³⁶. Theoretical calculations show that amino groups make energetically favourable interactions with the π electrons of aromatic rings, and predict the optimal position for the amino–nitrogen to be between 3.0 and 3.4 Å from the ring centre^{37,38}, close to that observed in the SH2 domain. These calculations suggest that such interactions are comparable in strength with conventional hydrogen bonds between non-charged groups (reviewed in ref. 39).

All other interactions with the phosphate involve the C-terminal end of strand 2 and the phosphate binding loop (between strands 2 and 3), which forms a flap that is almost perpendicular to the plane of sheet B and covers the phosphate group (Figs 2 and 3). This flap is on the very edge of the peptide binding surface, and is suggestive of a lid on the phosphate-binding site that might be in an open conformation in the absence of ligand. This orientation of the loop is stabilized by a strong hydrogen bond between the side chain of Glu 178 and the backbone amide nitrogen of Arg 155, which might also be an important factor in orienting the arginine at the binding site. Three of the residues in the loop interact directly with the phosphate group: the backbone amide of Glu 178, and the hydroxyl groups of Ser 177 and Thr 179 all form hydrogen bonds with phosphate oxygens. Threonine 180, which holds Lys 203 in place, is also in this loop and provides an additional indirect interaction with the phosphotyrosine.

Histidine 201 in strand 4 is strongly conserved among SH2 domains (Table 2), and it has previously been identified as potentially being part of the phosphate binding site. It does not, in fact, make any direct hydrogen-bonding interactions with the phosphotyrosine residue but does make contacts that may be critical for the maintenance of active-site geometry. Along with side chain atoms of Lys 203, the C β atom of His 201 forms part of the binding pocket for the tyrosine ring of the substrate. The carbonyl oxygen of the histidine makes the sole hydrogen bond with the peptide backbone. Finally, the histidine side chain makes two hydrogen bonds, with the side chains of Glu 159 in helix 1 and Ser 187 in strand 3 and this interaction may help optimize the orientation of helix 1. Ser 187 in turn forms a hydrogen bond with the N ϵ atom of Arg 175. Notably, mutation of His 201 to leucine in c-Src has little effect on the transforming properties of the protein¹⁹. Such a mutation would be expected to preserve both the interactions with the phosphotyrosine ring and the hydrogen bond with the substrate.

Arginine 175, which forms the ion pair with the phosphate group, is strictly conserved in the thirty SH2 domains that we have surveyed (Table 2). Arginine 155, which recognizes the aromatic ring, is conserved in all but three: the C-terminal SH2 domain of GAP, where it is replaced by lysine, and the two SH2 domains of a protein-tyrosine phosphatase, where it is replaced by glycine (Table 2). The strict conservation of Arg 175 can be rationalized in terms of the fact that it is completely buried and alternative positions do not appear to exist for placing groups to form bidentate ion pairs with the phosphate. Arginine 155, on the other hand, is on the surface and its function can potentially be replaced by a number of other residues and it is likely that alternative mechanisms have evolved for recognizing the ring system. For example, in the C-terminal SH2 domain of GAP the residue corresponding to His 201 in Src is replaced by an arginine. In Src SH2, the histidine ring stacks against the

guanidinium group of the side chain of Arg 155, which in the C terminal SH2 domain of GAP is a lysine. Simple model building suggests that the N ϵ atom of the lysine may interact only with the phosphate and not the ring system. However, an arginine replacing His 201 could readily form an amino–aromatic interaction with the phosphotyrosine ring system, and potentially interact with the phosphate as well.

Lysine 203, which forms both hydrophobic and amino–aromatic interactions with the phosphotyrosine ring, is not as strongly conserved as the two arginines. But it is replaced in all cases by residues that can potentially provide amino–aromatic interactions (arginine or histidine) or hydrophobic interactions (Table 2). Potential interactions between the phosphate group and the phosphate-binding loop appear to be conserved in sequences closely related to Src, but are difficult to evaluate in about half of the sequences analysed (Table 2).

In the peptide A SH2 complex, the side chain of Arg 217 from a symmetry-related molecule is in close proximity to the phosphate group. But in the peptide B complex there are no such crystallographically related molecules near the phosphotyrosine, and interactions very similar to those seen in the peptide A complex occur between the phosphotyrosine residue and the primary protein molecule (Fig. 4). These are therefore independent of the effects of crystal packing and the amino acids C-terminal to the phosphotyrosine, and are likely to reflect the intrinsic recognition mechanism utilized by Src SH2. One potential problem is that both peptides have N-terminal phosphotyrosines, and the positively charged N-terminal amino group is positioned close to the terminal nitrogens of Arg 155. This interaction is expected to be destabilizing, and is likely to be a factor contributing to the relatively low affinity of these peptides for Src SH2 domain.

Comparison with other SH2 sequences

All thirty SH2 sequences that we have examined can be aligned with no insertions or deletions within strands of sheet A and the two helices (Table 2). This results in the preservation of hydrophobic character at all 11 positions that have side chains in Src SH2 that are completely buried in a hydrophobic tertiary environment, strongly suggesting that all SH2 domains have similar three-dimensional structure. A comparison of 67 SH2 sequences and a prediction of the secondary structure of the domain has recently been published⁴⁰. This analysis is essentially correct in its predictions, although some elements of sheet B are missing and the boundaries of the secondary structural elements are not exactly placed. The variation in elements of sheet B is consistent with its position in the three-dimensional structure, away from the peptide binding surface and the central scaffolding. The putative SH2 domain in the serine/threonine kinase encoded by the oncogene *akt*⁴¹ does not fit into our alignment of SH2 sequences, consistent with the results of the prediction analysis⁴⁰.

Eisenberg and co-workers have recently developed a profile method of sequence alignment that is based on the availability of the three-dimensional structure of at least one member of a family of sequences⁴². We have used this method to rank SH2 sequences in terms of their structural similarity to Src SH2 (Table 2). The top six sequences (products of *yes*, *hck*, *fgr*, *fyn*, *lck* and *lyn*; all of the *src* family) are very closely related to Src SH2, with no insertions or deletions within the entire domain. Sequence identities for these range from 60% to 70%, and they are likely to be essentially identical to Src SH2 in three-dimensional structure. Several of the sequences at the other end of the scale have correlation (Z) scores lower than 7, the value that is required to detect structural similarity in studies of other proteins⁴². Thus, although these sequences can be unambiguously identified as SH2 domains⁴⁰, they are likely to contain significant, and potentially important, variations on this structural theme.

Regions of extensive variability (the loops connecting strands

3, 4, 5 and 6, and the loop extending from the C-terminal end of helix 2) occur on or close to the peptide binding surface (Fig. 3). These regions can potentially interact with proteins presenting phosphotyrosine residues to the SH2 domain, and are likely to contain the elements that determine sequence specificity and differential binding. Site-specific mutations and deletions targeted to these regions are unlikely to have major consequences for either the global fold of the domain or its ability to bind phosphotyrosine, and such modifications should aid greatly the more specific definition of the elements of substrate specificity.

Discussion

The SH2 domain contains a novel scaffolding for presenting a peptide-binding surface. Previously determined peptide or protein-binding structures include proteases (with the binding site between two subdomains), immunoglobulins (with loops extending from a β -barrel and forming the binding site) and the major histocompatibility complex (with peptide bound between two surface helices)⁴³. In contrast, the binding surface

of the SH2 domain is perpendicular to a β -sheet and contains no deep crevices or depressions. This structure may be compared figuratively to a right hand, with the fingers corresponding to sheet A and the phosphotyrosine grasped between the tip of the thumb (helix 1) and the tips of the fingers (compare with Fig. 3). A simple explanation for the discrimination between phosphotyrosine and phosphoserine or phosphothreonine is apparent: a phosphate group attached to the shorter sidechains would be unable to reach into the binding pocket and interact directly with the buried arginine.

Inhibition of SH2 domains is likely to have interesting consequences for the ability of cells to respond to stimulation by growth factors and for the transforming properties of oncogene products. The structure presented here provides obvious suggestions for the design of general SH2 inhibitors, as well as some clues for extending the specificity to particular SH2 domains. The structure also defines those elements of the sequence that are required for maintaining the integrity of the three-dimensional fold, and hence provides a basis for the design of proteins with additions or deletions to the basic architecture. □

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Evidence for single-temperature dust in the Crab nebula from a reanalysis of its infrared spectrum

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THE Crab nebula, the remnant of the celebrated supernova of 1054 (refs 1, 2), lies 2 kpc from the Earth^{3,4} and is the most powerful neutron-star-driven nebula known. Its emission from radio to X-ray wavelengths is predominantly synchrotron radiation, with a power-law spectrum that steepens abruptly at 10^{13} and 10^{16} Hz (ref. 4). The infrared satellite observatory IRAS revealed significant excess emission, above the synchrotron spectrum, peaking between 60 and 100 μm in wavelength⁵. This was attributed to thermal radiation by dust with at least two characteristic temperatures in the range 40–100 K (refs 5, 6). We have now reanalysed the IRAS data, taking care to remove contamination by background emission, and find that the revised infrared flux densities are in fact well explained by a single dust component at a temperature of 46 K. The required dust mass is 0.02 solar masses ($M_{\odot}$), corresponding to a gas to dust ratio of 100:1. We also determine more accurately the break frequency, 1.4×10^{13} Hz, in the power-law spectrum. This value implies, for a steady-state synchrotron model, a time-averaged magnetic field of 420 μG , which is less than the value corresponding to an equipartition of energy between radiating particles and magnetic field, but probably greater than the present field strength.

Since the publication of IRAS flux densities for the Crab nebula⁵, additional survey data have become available in the form of hours-confirmed coverages (HCONs) of the sky, from which 'Skyflux' maps were constructed⁷. We have reanalysed the three existing HCONs to obtain a comprehensive determination of the Crab nebula flux density for each survey band and an estimate of the appropriate uncertainty. In particular, we have taken care to determine and remove background emission, which is dominated by zodiacal dust at the two shorter wavelengths, and cooler galactic dust at the longer ones. Colour corrections (the ratio of the 'Skyflux' intensity in each band to the value at the band centre) have been calculated by integrating model spectra over the IRAS bands. The error in the final determination depends on the spread in the HCON values (the dominant contribution) as well as on uncertainties in correcting for the background. The approach is similar to that used in analysing IRAS observations of the southern supernova remnant RCW86 (ref. 8), except that the relative compactness and strength of the Crab nebula make it easier to separate its emission from the background components.

The final flux densities that we have obtained for the four IRAS wavelengths are listed in Table 1. Previously published intensities^{5,9} were based on only one HCON; our values are generally in good agreement with those found by Arendt⁹ and, within the stated errors, are consistent with the findings of Marsden *et al.*⁵ except at 25 μm . The new flux density in this band is 30% lower than their value, and we can find no apparent reason for this discrepancy; the cause may lie in the background removal, the way a correction for source extent was applied, or both. The 6–333- μm spectrum of the Crab nebula with the new data points is shown in Fig. 1. Our 12- μm flux density is lower

than both the original value⁵ and an extrapolation from shorter wavelengths⁴. It can be used in determining the infrared-optical-ultraviolet spectrum. Woltjer⁴ has reassessed the all-important extinction correction and argues that the most plausible spectrum is either straight or gradually steepens with frequency. He shows that the existing data are consistent with a single power law, of spectral index $\alpha \approx -0.85$ (defined from $S \propto \nu^{\alpha}$, where S is the flux density in Jansky and ν the frequency in Hertz), provided the colour excess is $E(B-V) \approx 0.4$ mag. We find that values of $E(B-V) = 0.37$ – 0.42 mag give an acceptably smooth spectrum, with α ranging from -0.88 to -0.80 . Because the steepening observed between the radio-millimetre (where $\alpha = -0.3$) and infrared-optical regions is close to 0.5, the value expected from simple radiation losses with continuous particle injection¹¹, we adopt $\alpha = -0.8$, thereby assuming $E(B-V) = 0.42$ mag. (The result of this fit to the data gives $S = 1.99(\nu/10^{15})^{-0.80}$ Jy, for $2 \times 10^{13} \text{ Hz} < \nu < 3 \times 10^{15} \text{ Hz}$; the line is plotted in Fig. 1.)

Excess emission is only present at the three longer IRAS wavelengths. We have analysed it by first subtracting the non-thermal power-law contribution⁴ from the total flux densities in Table 1. The peak lies near 70 μm and has a width that is difficult to emulate by nonthermal emission mechanisms⁶: even monoenergetic electrons radiating in a perfectly uniform magnetic field produce very broad-band emission¹¹ (see Fig. 2), and there is likely to be a spread in both particle energy and field strength. The addition of even a weak nonthermal component (Fig. 2) considerably broadens the excess and reduces the quality of the fit, leading us to conclude that any nonthermal contribution to the excess is minor. The best-fitting thermal component emitted by dust with an absorption efficiency as modelled by Draine and Lee¹³ has a temperature of 46 ± 3 K. (The less realistic case of pure black-body emission produces a peak significantly broader than the excess in Fig. 2). The new (lower) flux densities that we find at 12 and 25 μm obviate the need to postulate additional high-temperature dust emission^{5,6}. The total luminosity in the infrared excess is $\sim 890 \pm 50$ times the solar luminosity $L_{\odot}$ (for an assumed distance of 2 kpc). This is emitted by $2.2 \pm 0.7 \times 10^{-2} M_{\odot}$ of dust, which combined with an estimated 1–3 $M_{\odot}$ for the shell^{4,14} suggests a gas:dust ratio close to the 100:1 canonical value. The proportion of shocked dust is, however, rather higher than in the other young supernova remnants RCW86, Cas A, Tycho, Kepler and SN1006 (ref. 8),

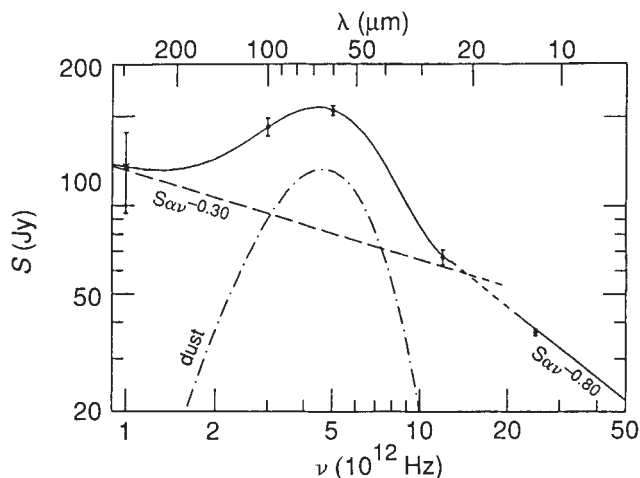
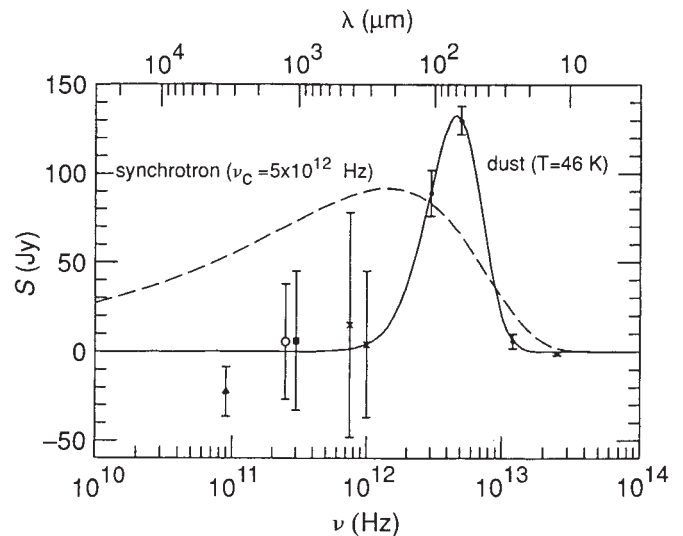


FIG. 1 The spectrum of the Crab nebula from 0.9×10^{12} to 50×10^{12} Hz (333 to 6 μm). Dashed lines show the best-fitting power-law extrapolations as indicated. The flux densities at 12, 25, 60 and 100 μm (●) are from Table 1, and a 300- μm value¹⁰ (×) is also shown. The curve is the emission expected from dust at 46 K (shown separately) added to the power law.

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FIG. 2 The infrared excess, shown with the best-fitting dust component. The flux densities at 12, 25, 60 and 100 μm (●) are from Table 1, with excesses at 300 and 400 μm (×) from Wright *et al.*¹⁰, at 1 mm (■) from Chini *et al.*¹², at 1.2 mm (○) from Mezger *et al.*⁶ and at 3.3 mm (▲) from C. J. Salter *et al.* (personal communication) also indicated. In addition, the excess expected from monoenergetic synchrotron particles (whose energy corresponds to emission at $\nu_c = 5 \times 10^{12}$ Hz) radiating in a uniform magnetic field is shown (dashed line).



by a factor of 10 or more. Either there is an intrinsically lower dust ratio in these young shell remnants, or most of the dust component must be in a state that prevents it from radiating much at infrared wavelengths.

The Crab nebula consists of an amorphous nonthermal component enveloped in a (thermal) network of line-emitting filaments. The two components are spatially distinct, as can be demonstrated from the filaments' kinematics³. The dust responsible for the infrared excess is most probably associated with the outer nebular shell. Some support for this is found in excess extinction which has been shown to coincide with several prominent filaments¹⁵. These results have been confirmed by more-detailed studies^{16,17} which reveal, however, that the dust is unevenly distributed among even the bright filaments. Although the amount of dust construed from these observations seems to fall short of that required to account for the infrared excess, the mass could be underestimated if the dusty cores have very high densities, if a significant diffuse dust component is associated with the fainter filaments or if the assumed grain size is too small¹⁶. High-resolution observations near 70 μm would be required to see how well the excess emission correlates with the thermal filaments.

Almost all of the infrared excess is emitted at wavelengths longer than that of the break in the power-law spectrum, which is further evidence that the two phenomena are unrelated. Because the 25- μm flux density shows some excess (see Fig. 2), it is likely that the spectrum steepens somewhere between 12 and 25 μm . The extrapolated power-law spectra meet at 1.45×10^{13} Hz (Fig. 1). Considering the uncertainties in extrapolating the long-wavelength spectrum in particular, we are confident that the break falls in the range $(1.2-1.8) \times 10^{13}$ Hz. As noted above, the change in spectral index on either side of the break is about $\Delta\alpha = -0.5$, the value expected from radiation losses¹¹. In the case of synchrotron loss (assuming a static, steady synchrotron source), the break frequency ν_b (Hz) is related to the

age of the emitting particles τ (s), the time-averaged magnetic energy density (responsible for the break in the energy spectrum) and the present field strength B_p (which determines the break frequency)

$$\left[\frac{\int B^2(t) dt}{\int dt} \right]^2 = \langle B^2 \rangle^2 = B_p^2 \frac{1.32 \times 10^{24}}{\nu_b \tau^2} \quad (1)$$

(where we define the break to be the point of intersection of the two power-law spectra and have assumed an average particle pitch angle of 60°).

The detection of γ -ray emission at teraelectronvolt energies produced by inverse Compton scattering¹⁸ provides a direct measure of B_p , namely $\sim 300 \mu\text{G}$. (Estimates of the equipartition field strength¹¹ give $B_{eq} \approx 500-1,000 \mu\text{G}$; see for example ref. 19.) Equation (1) then gives

$$\langle B^2 \rangle^{1/2} = 420 \pm 15 \mu\text{G}$$

The estimate of B from equation (1) assumes only radiation losses, whereas a more thorough treatment takes into account the effects of expansion²⁰⁻²². It is nonetheless intriguing that $B_p \approx \langle B \rangle^{1/2} \approx B_{eq}$. Is their near-equality just a coincidence? $\square$

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TABLE 1 Infrared flux densities

λ (μm)	Flux density (Jy)		
	total	error	excess*
100	184	13	89
60	210	8	130
25	67	4	6
12	37	1	-1

* After removal of nonthermal power-law components.

Stable photoinduced charge separation in layered viologen compounds

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SOLAR energy can be used and stored by the efficient production of long-lived photoinduced charge separation¹⁻³—a state achieved in photosynthetic systems by the formation of a long-lived radical pair⁴⁻⁷. A number of artificial systems have been reported that efficiently undergo photochemical charge transfer⁸⁻¹⁰; unfortunately, the thermal back electron transfer often proceeds at an appreciable rate, limiting the utility of these systems. Here we report the photochromic behaviour of novel layered zirconium phosphonate/viologen compounds which show very efficient photoinduced charge transfer, and form a charge-separated state which is long-lived and stable in air. Spectroscopic studies indicate that the photoproduct is the dialkyl viologen radical cation, produced in the interlamellar region of the zirconium phosphonate. We suggest that the remarkable stability of the charge-separated state arises from structural features which allow for stabilization of the radicals by delocalization and shielding from molecular oxygen.

Production of long-lived photoinduced charge separation is an important goal in the development of systems for solar energy conversion and storage¹⁻³. The charge separation in these systems typically involves a redox reaction between a photoexcited donor and a suitable acceptor, resulting in the production of radical ion pairs (equation (1)).



The cation and anion generated in this way are better oxidants and reductants, respectively, than either of the neutral ground-state molecules. To harvest the light energy put into this system, the oxidizing and reducing power of the photogenerated species must be used before the electrons are transferred back (equation (2)), regenerating the starting materials. A considerable challenge is to control this thermally fast back electron transfer reaction, which is photochemically unproductive. Its rate has been decreased to some extent by incorporating the acceptors and donors into solid matrices^{9,11-18}. The lifetimes of the charge-separated states in these heterogeneous systems range from seconds to hours.

There has been particular interest in the dialkyl viologens (*N,N'*-dialkyl-4,4'-bipyridinium dihalides) as photochemical electron acceptors because the viologen radical cations produced in this manner can subsequently be used to reduce protons to hydrogen^{19,20}. Dialkyl viologens can behave as one or two electron acceptors and can be reduced both chemically^{21,22} and photochemically²³⁻²⁶. The one-electron reduced product is the well-characterized blue radical cation, which is oxidized rapidly by molecular oxygen and other radical scavengers^{18,27}. Incorporation of methyl viologen into zeolites¹¹, clays²⁸, and other molecular assemblies¹⁶⁻¹⁸ has led to changes in its photochemical reactivity and in some cases to greater radical cation stability.

Layered zirconium phosphonate/viologen compounds (ZrPV(X), where X is Cl, Br) were prepared as shown in Fig. 1. Tetravalent metal phosphonates crystallize with structures similar to α -Zr(O₃POH)₂·H₂O (α -ZrP)²⁹. Each layer consists of a plane of metal atoms linked together by phosphonate groups. The metal atoms are octahedrally coordinated by oxygen atoms, with the three oxygens of each phosphonate tetrahedron

bound to three different metal atoms. This arrangement forces the organic groups to lie above and below the inorganic layer. For diposphonic acids, similar to PV(X), the organic moiety is bound to adjacent metal planes, as shown schematically in Fig. 1. The peak positions in the X-ray powder diffraction patterns of ZrPV(Cl) and ZrPV(Br) are identical, suggesting that the two compounds have identical unit-cell parameters. If the halides were directly associated with the faces of the viologen groups, as observed in crystalline viologen dihalides³⁰, the unit-cell sizes for the Cl and Br compounds would be different. A more likely structure has the halide counterions in octahedral sites on the surface of the zirconium phosphonate lamellae (Fig. 1). These sites are the same as those occupied by the water molecules of α -ZrP³¹.

When ZrPV(Cl) is exposed to solar radiation or to a medium-pressure mercury arc lamp, the white solid immediately turns blue. The blue colour persists indefinitely in air and remains

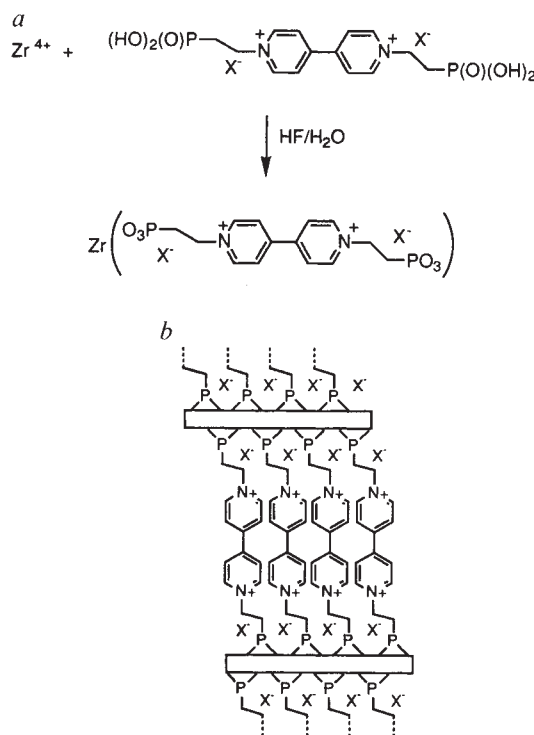


FIG. 1 *a*, Synthesis of zirconium phosphonate/viologen compounds (ZrPV(X), where X is Cl, Br) from Zr(SO₄)₂ and the appropriate viologen diposphonic acid (PV(X), X is Cl, Br)⁴⁸⁻⁵⁰. (The diposphonic acids (PV(X), where X is Cl, Br) are prepared by reaction of BrCH₂CH₂P(O)(OCH₂CH₂)₂ with 4,4'-bipyridine in water. The ester is hydrolysed with aqueous HCl or HBr to give the chloride and bromide, respectively. The diposphonic acids are crystallized from isopropanol/water.) The ZrPV(X) crystallites formed in this reaction are $\leq 1 \mu\text{m}$ in size (as seen by scanning electron microscopy). *b*, Proposed structure for ZrPV(X). Bars represent the phosphonate oxygen and zirconium atoms. Spectroscopic and thermal data: infrared (KBr), PV(Cl): 3,112, 3,014, 1,640, 1,555, 1,506, 1,443, 1,358, 1,281, 1,175, 1,112, 1,020, 936, 816, 485 cm⁻¹; ZrPV(Cl): 3,119, 3,049, 1,633, 1,563, 1,506, 1,443, 1,386, 1,161, 1,041, 865, 823, 767, 731, 539, 499 cm⁻¹. ¹³C NMR (d₆-acetone) (PV(Cl): 30, 58, 128, 147, 151 p.p.m. Cross-polarization magic angle spinning ¹³C NMR ZrPV(Cl): 29, 66, 129, 147 p.p.m. Ultraviolet spectra by diffuse reflectance, λ_{max} PV(Cl): 295 nm; ZrPV(Cl): 295 nm; PV(Br): 325 nm; ZrPV(Br): 325 nm. CHN analysis (%) by standard flash combustion techniques: ZrPV(Cl) calculated: C, 31.59; H, 3.03; N, 5.26; observed: C, 30.9; H, 3.8; N, 5.2. Low CHN numbers are often observed for zirconium phosphonates, because the organic material gets trapped and slowly burns. If the solid is heated for 10 hours at 1,000 °C in the air, the organic material burns out completely leaving ZrP₂O₇ (ref. 51). The expected weight loss in this transformation is 50.2% (ZrPV(Cl)=532 g mol⁻¹ and ZrP₂O₇=267 g mol⁻¹); the observed weight loss is 50.1%. For ZrPV(Br), the expected weight loss is 57.3%; the observed weight loss is 57.5%.

after the light source has been removed. The colour fades gradually and is removed completely only by dissolution in HF or by heating to 100 °C. The photolysed material has been characterized by diffuse reflectance ultraviolet-visible spectroscopy; it shows peaks at 295 and 400 nm, and a broad absorption centred at 600 nm (Fig. 2). This spectrum is similar to that found for reduced viologen in other inorganic matrices^{11,16}, and to the solution spectrum of chemically reduced PV(Cl) (reduced with aqueous basic sodium dithionite; peaks at 285, 384 and 600 nm). The electron spin resonance (ESR) spectrum of this blue solid gives a single resonance at $g = 2.005$, peak width 8 gauss. Photolysis of ZrPV(Br) produces a blue solid whose electronic spectrum is similar to that of photolysed ZrPV(Cl) (Fig. 2). This blue solid is also ESR active. Diffuse reflectance and ESR spectra indicate that ZrPV(Br) is less reduced than ZrPV(Cl) under identical photolytic conditions.

ZrPV(Cl) and ZrPV(Br) also react with chemical reducing agents. Reaction with basic dithionite gives a blue solid, but cleavage of the carbon-nitrogen bond occurs and the solid eventually dissolves. If the blue solid is filtered off before dissolution, the blue colour persists indefinitely under argon, but the solid immediately turns white on exposure to air. For the photolysed materials, oxidation by reaction with molecular oxygen is difficult, because oxygen is too large to diffuse into the solid. Similarly, reduction by chemical reducing agents, which are also too large to diffuse through the layers, may result in reduction of only those viologens which are exposed to the surface. This is the most likely explanation of why the chemically reduced species is rapidly decomposed in air and the photochemically reduced material is not.

The lack of fine structure in the ESR spectrum is most probably because the free electron is delocalized over more than one viologen³². Hyperfine coupling to both H and N is observed for viologen reduced in fluid solution or frozen glass matrices^{33,34}. A single sharp line, similar to the one observed here, is reported for reduced viologens adsorbed on cellulose³⁵ and for viologen radical aggregates formed at high concentration and low temperature³⁴. There is no evidence in the electronic spectra for the formation of diamagnetic dimers, which are formed when reduced viologen is present in high concentration³⁶⁻³⁸.

The photoaction spectrum for photoinduced charge transfer in ZrPV(X) is shown in Fig. 3. The wavelength of the peak in the photoaction spectrum is very close to that observed in the

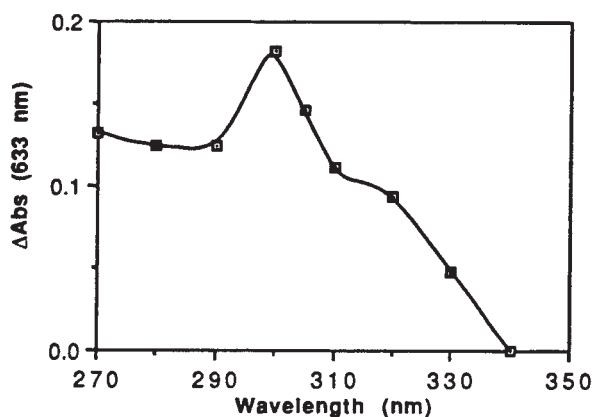


FIG. 3 Photoaction spectrum of ZrPV(Cl). Samples of ZrPV(Cl) were irradiated with a 150-W xenon lamp equipped with a monochromator. The samples consisted of ZrPV(Cl) pressed into a KBr pellet. The photochemical reduction occurred only at wavelengths ≤ 330 nm (45 min irradiation, slit set to give beam ± 10 nm of the central wavelength). The photoproduct was detected by measuring the change in intensity (I) of a 1-mW Aerotech helium-neon laser (633 nm) transmitted through the pellet before and after photolysis $\Delta\text{Abs} = \log(I/I_0)$.

diffuse reflectance spectrum of ZrPV(Cl) (Fig. 2). This transition has been reported to be due to charge transfer from the halide counterion to the viologen^{39,40}. The quantum yield for this photochromic reaction in ZrPV(Cl) has a lower limit of 0.03 (at 300 nm). The main source of error in measuring the quantum yield was in determining how much of the incident light was absorbed and how much was scattered by the sample. The anisotropic nature of the compound made it impossible to eliminate scattering with an index-matched fluid. A lower limit for the scattering loss at 300 nm was estimated to be 90%, on the basis of measurements made at longer (nonabsorbing) wavelengths. Solar irradiation of ZrPV(Cl) with a 350–600-nm band-pass filter or a 450-nm cutoff filter gave no photoreduction. This indicates that the photochemistry occurring with solar radiation is due solely to the small proportion of short-wavelength solar light. ZrPV(Cl) gives an emission spectrum with a maximum at 300 nm, on irradiation at 285 nm. ZrPV(Br) did not show any emission. Viologens have not been observed to emit in solution but viologen-intercalated clays have been reported to fluoresce with an emission spectrum similar to that given by ZrPV(Cl)²⁸.

We also prepared a methyl viologen intercalation compound of α -ZrP. We first treated α -ZrP with $(\text{C}_4\text{H}_9)_4\text{N}^+\text{OH}^-$ to give $[(\text{C}_4\text{H}_9)_4\text{N}^+]_{0.3}[\text{Zr}(\text{O}_3\text{POH})_{1.7}(\text{O}_3\text{PO}^-)_{0.3}]$. Methylviologen was then ion-exchanged for the tetrabutylammonium ions. The interlayer spacing observed ($d = 10.6$ Å) suggests that the methyl viologen molecules are oriented parallel to the plane of the α -ZrP layers. In this system, there are no halide counterions present, because the host serves as a macroanion which balances the charges on the viologens. No photochemical viologen reduction was observed for this system. This observation supports the premise that the photochemistry is due to charge transfer from the halide counterions. In addition, the lack of photochemistry in the ion-exchanged material eliminates the possibility that the photochemistry is occurring because of metal impurities in the starting materials.

The diffuse reflectance and ESR spectra indicate that the photoproduct is the dialkyl viologen radical cation, produced in the interlamellar region of the zirconium phosphonate. The photoaction spectrum indicates that charge transfer is occurring from the halide counterions to give the viologen radical cation. Charge transfer from the counterions of dialkyl viologen dications has been reported to occur in solution, but is followed by rapid back electron transfer to regenerate starting materials²⁴. In this case, back electron transfer undoubtedly occurs, but an

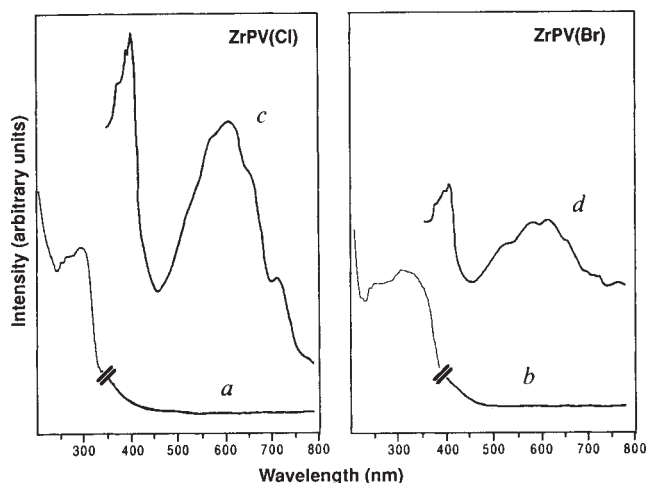


FIG. 2 Electronic spectra of ZrPV(Cl) and ZrPV(Br). *a, b*, Ultraviolet-visible spectra of ZrPV(Cl) and ZrPV(Br), respectively. *c, d*, Visible spectra of ZrPV(Cl) and ZrPV(Br), respectively, after photolysis with a medium-pressure mercury arc lamp. All spectra were measured by diffuse reflectance on powdered samples. The double bar between 350 and 400 nm represents a lamp change. Relative intensities of the spectra from the two different lamps are meaningless.

alternative reaction path must be available in which the halide radical becomes trapped. This trapping mechanism must be competitive with back electron transfer in order to lead to the observed product. The trapping could be in the form of a X_2^- ion (the absorption bands for X_2^- could be hidden under the 400-nm absorption)⁴¹, but this seems unlikely as diffusion of $X^\cdot$ or X^- should be slow in this close-packed lattice. Another possibility is that the photogenerated halide radical removes an electron from the ZrO_6 system. This would correspond to oxidizing one of the phosphonate oxygens, with the positive charge being delocalized over all six Zr bound oxygens. We are investigating the mechanism of this photochemical conversion and the nature of the charge-separated state by preparing other $ZrPV(X)$ and $MPV(X)$ (X is iodide or pseudo halide, M is group 4 or 14 metal ion) compounds, and studying their photochromic behaviour.

The photoactive species must be optimally oriented on both molecular and crystallographic scales to produce a usable photochromic system⁴². The observed quantum yield and long lifetime of the coloured state of $ZrPV(Cl)$ demonstrate that the photoactive species in this compound are well ordered. Unfortunately, this material would not be useful for solar energy storage or conversion, because of the close-packed nature of the interlamellar region: substrates cannot reach the photoreduced viologen to extract the stored photochemical energy. A related system overcomes this diffusion problem by encasing the viologen in a porous sol-gel glass¹⁴. Substrates can then readily diffuse in and react with the photoreduced viologen. Mallouk *et al.*⁴³ have reported a system in which the photoreduced species is held in zeolite L, which also allows the diffusion of substrates to the photoreduced centres⁴³. We are currently investigating ways to make our zirconium phosphonates porous to allow for the diffusion of substrates to the photochemically reduced viologen. Porosity can be obtained by synthesizing mixed phosphonates⁴⁴⁻⁴⁷ (such as $Zr(PV(X))_{1-x}(O_3PCH_2CH_3)_{2x}$), where one of the phosphonates will be photochemically inactive. The $PV(X)$ unit will act as a pillar holding the layers apart, making the material porous and allowing access to the reduced viologen. □

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Spectroscopic observation of a catalyst surface in a reactive atmosphere at high pressure

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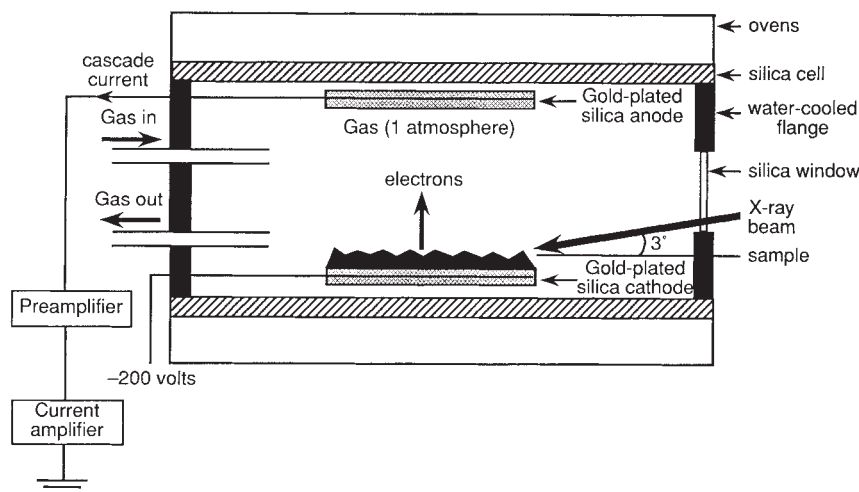
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A PRINCIPAL objective in the study of heterogeneous catalysis is the determination of the structure and composition of solid surfaces under reaction conditions. Most applicable spectroscopic methods achieve surface sensitivity by detecting ejected electrons which have very short mean free paths in the solid¹. But such measurements must be carried out in vacuum, whereas catalytic reactions are typically carried out at pressures of at least one atmosphere. Although such a gas environment can be penetrated by X-rays or neutrons, these probe the bulk properties of solids rather than their surface properties²⁻⁶. Here, we describe how a catalyst sample and its gas environment may be configured so as to constitute a photocathode ionization detector⁷. By monitoring the ionization cascade rather than photon absorption, we show that X-ray absorption spectroscopy can be used to examine monolayer amounts of adsorbate on the surface of a practical catalyst at one atmosphere pressure. The wider application of this technique to complex systems in a variety of environments has considerable potential for the study of heterogeneous catalysis, and may be extended to other areas of interfacial science.

We investigated an industrial copper-containing catalyst for methanol synthesis from $CO/CO_2/H_2$ mixtures. (The extent to which the copper surface is covered by oxygen under reaction conditions is the subject of controversy^{4,8}.) Experiments were done on station 8.1 at the SERC Daresbury synchrotron using a specially designed cell constructed of silica and stainless steel (G.D.M. and T.R., manuscript in preparation; see Fig. 1). The catalyst (60% CuO, 30% ZnO, 10% Al_2O_3) has previously been reduced in H_2 and subsequently passivated in N_2O . Samples of this material were deposited on a gold-coated silica plate which served as a cathode. Under X-ray irradiation, photoemitted electrons from the sample caused an ionization cascade in the ambient gas (pure He or reactive mixtures containing H_2 and CO_2). This cascade was detected at a gold-coated silica anode: it provides a measurement of X-ray absorption by the sample. By recording the cascade current (electron yield) as a function of X-ray wavelength, one obtains the X-ray absorption spectrum: this contains structural information about the environment of the photoemitting atom². The essential point is that we monitor absorption of photons by measuring the flux of photo-ejected

FIG. 1 Schematic of the surface-EXAFS cell.



electrons. The X-rays penetrate deep into the solid whereas the electrons undergo strong inelastic scattering on their way out of the sample. This is the effect we exploit to obtain X-ray absorption data pertaining to the surface of the solid: the sampling depth now depends on the mean free path of the ejected electrons (a few atomic layers) rather than the penetration depth of the X-rays (of the order of micrometres). For example, photo-ejected primary electrons with energies of $\sim 50\text{--}350\text{ eV}$ would have a mean free path of 2–4 atomic layers. The experiments were carried out at the Cu K absorption edge (photon energy $\sim 9,000\text{ eV}$) and should therefore provide information about the environment of surface Cu atoms (co-ordination and bond lengths).

Figure 2 illustrates the radial distribution functions (RDF) produced by Fourier-transforming the resulting extended X-ray absorption fine structure (EXAFS) data. Results are shown for the starting catalyst, after reduction in H_2 , after treatment with a $\text{CO}_2/\text{CO}/\text{H}_2$ mixture and after treatment with CO_2 at 523 K (reaction temperature). Also shown are results obtained with a copper foil standard. The RDFs of both the reduced catalyst and the catalyst treated in $\text{CO}_2/\text{CO}/\text{H}_2$ are almost identical to the RDF obtained with a standard copper foil. Treatment with CO_2 , however, produces clear evidence for the presence of Cu–O bonds at the surface (radius $r = 1.849\text{ \AA}$). Comparison of this RDF with those for samples of bulk Cu_2O and CuO (ref. 9) clearly indicates that our data do not reflect the formation of a bulk oxide phase. Following CO_2 treatment, the Fourier transform peak corresponding to the first coordination shell in copper metal is broadened towards longer distances; this is consistent with either a mixture of Cu_2O and Cu being sampled or significant disruption of the copper lattice on oxidation of the outer layer.

The key question is how much oxygen this represents: a monolayer, or a layer of oxide many layers thick. The surface sensitivity of the technique and our ability to observe adsorbed layers under other conditions depend on the answer to this question. To resolve this issue, we used X-ray photoelectron spectroscopy (XPS) and X-ray diffraction (XRD).

XPS measurements were made on a Cu(111)-oriented single crystal specimen in order to obtain an estimate of the oxygen surface coverage under the conditions of the electron yield measurements on the practical catalyst. The instrumental sensitivity was calibrated by exposing a clean Cu(111) single crystal to O_2 gas under conditions known to produce ~ 0.4 monolayers of chemisorbed oxygen atoms¹⁰. A clean metal surface was then regenerated and exposed to 80 Torr CO_2 at 523 K; these conditions are identical to those used for the electron yield measurements. Measurement of the $\text{O}(1s)$, $\text{Cu}(2p)$ and $\text{C}(1s)$ emission showed that this procedure resulted in deposition of ~ 0.5 monolayers of adsorbed oxygen ($\text{CO}_{2(g)} \rightarrow \text{O}_{(a)} + \text{CO}_{(g)}$).

A possible objection to this control measurement is that macroscopic Cu crystals may not have the same reactivity as the small Cu particles present in the catalyst: the latter may undergo gross oxidation by CO_2 to produce particles of copper oxide. To check this important point, we made *in situ* XRD measurements on the catalyst in gas environments corresponding exactly to those used for the electron yield work. Figure 3b shows a difference pattern produced by subtracting the XRD pattern due to the CO_2 -treated catalyst from that due to the H_2 -reduced catalyst. CO_2 treatment leads to a $\sim 12\%$ attenuation of the Cu(111) reflection. As the Cu particle diameter in this material is $\sim 105\text{ \AA}$, the estimated thickness of the metal layer

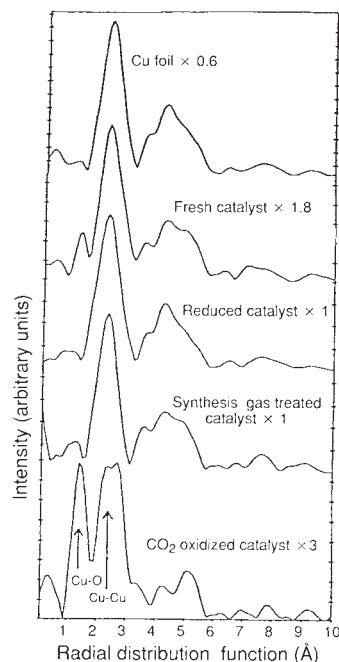


FIG. 2 Radial distribution functions (RDFs) derived from electron yield EXAFS data. From top, Cu foil (in transmission); fresh catalyst (60% Cu, 30% ZnO, 10% Al_2O_3 , reduced in H_2 and passivated in N_2O); catalyst treated in (10% H_2 /90% He) at 473 K for 1 hour; catalyst treated in (1% CO /1% CO_2 /8% H_2 /remainder He) at 503 K for 0.5 hours; catalyst treated in (10% CO_2 /90% He) at 523 K for 0.5 hours. In each case, total pressure was $\sim 1\text{ atm}$. Data were analysed using the EXCURVE 90 package at Daresbury. In modelling these RDFs, bond lengths and coordination numbers for pure face-centred cubic Cu and Cu_2O were used. (The coordination numbers for the reduced and synthesis-gas treated catalysts are consistent with the 12, 6, 24 and 12 of the first 4 shells of copper metal; values of 3 ± 1.5 for Cu–O and 4 ± 2 for Cu–Cu may be obtained for the CO_2 -treated catalyst, depending on the Debye–Waller factors chosen.)

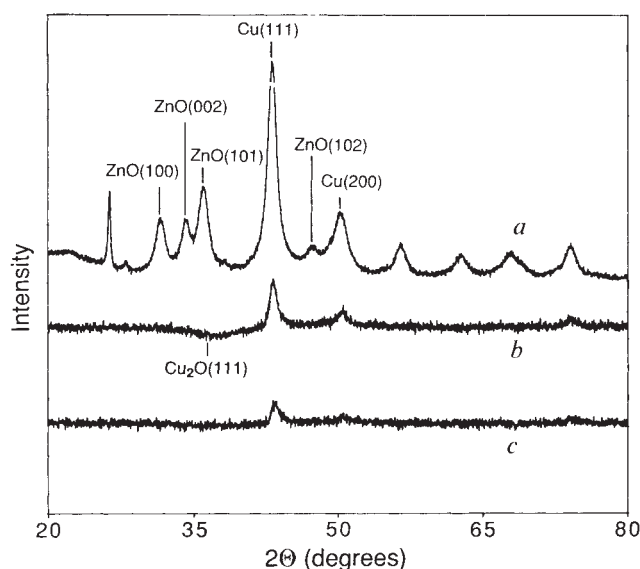


FIG. 3 *a*, Powder X-ray diffraction pattern for methanol synthesis catalyst treated in (10% H_2 /90% He) at 473 K for 1 hour. *b*, Difference spectrum obtained by subtracting XRD pattern of catalyst treated in (10% CO_2 /90% He) at 523 K for 1 hour from the pattern shown in *a*. *c*, Difference spectrum obtained by subtracting XRD pattern of catalyst treated in N_2O (298 K for 3 min) from the pattern shown in *a*. XRD patterns were collected over 10 h using a Siemens diffractometer. In each case total pressure was ~ 1 atm.

disrupted by CO_2 treatment (for example, by formation of an oxide film) is less than or about one monolayer. The reduction in the magnitude of the Cu first shell in both the as-received and CO_2 -treated catalyst can be rationalized in terms of such a disruption of the Cu lattice. It might be argued that the effects shown in Fig. 2 could be due to Cu particles that are too small to be detectable by XRD. This possibility was eliminated by quantitative control experiments which showed that at least 95% of the Cu in the catalyst sample was detectable by XRD.

A final caution is necessary. If there were an epitaxial relationship between the Cu metal and the oxidized surface layer in the catalyst particles, the calculated amount of oxidation could be an underestimate: Cu atoms in an overlying oxide layer could contribute to the Cu(111) intensity by Bragg interference¹¹. This possibility was checked by doing control experiments with N_2O , the standard titrant for Cu surface area measurements. The Cu- N_2O system has been well studied^{8,12}, both for macroscopic crystals and for small particles. At 300 K, the reaction produces a maximum oxygen uptake corresponding to ~ 0.5 monolayers of chemisorbed oxygen ($\text{N}_2\text{O}_{(\text{g})} \rightarrow \text{O}_{(\text{a})} + \text{N}_{2(\text{g})}$). Figure 3c shows the XRD difference pattern obtained following exposure to N_2O . Note that the Cu(111) intensity diminishes by $\sim 8\%$, because of out-of-phase X-ray scattering by both an overlayer of oxygen atoms and a slightly relaxed copper surface¹³.

Thus the XPS and XRD results indicate that CO_2 treatment of dispersed Cu at 523 K and 80 Torr leads to formation of a partial monolayer of surface oxide or chemisorbed oxygen. This adsorbed layer is readily detectable by electron yield EXAFS in the presence of a reactive gas environment. The extent to which the copper surface of a methanol synthesis catalyst is covered by oxygen under reaction conditions the subject of controversy^{4,8}. A comparison of the RDFs in Fig. 2 indicates that this coverage amounts to ≤ 0.1 monolayer of chemisorbed oxygen. This is consistent with previous work by Clausen *et al.*⁴ and Chinchin *et al.*⁸ who have investigated the bulk structures of working methanol synthesis catalysts. □

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Bromoform emission from Arctic ice algae

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DESTRUCTION of surface ozone in the Arctic environment during the spring is thought to be caused by photochemical reactions involving bromine compounds¹. Berg *et al.*² reported a pulse of bromine particles and gases in the Arctic lower atmosphere in spring, which may be responsible for this surface ozone destruction and for which biogenic sources have been hypothesized^{1–3}. Here we report laboratory and *in situ* measurements which indicate that Arctic ice microalgae emit significant quantities of bromoform (CHBr_3), which may be converted photochemically into active forms of bromine. Our estimates of total annual bromoform release indicate that polar ice algae might contribute globally significant amounts of organic bromine compounds, comparable with anthropogenic and macrophyte sources.

Berg *et al.*² suggested that the spring pulse of bromine might be caused by bromoform emission by red benthic macroalgae. Wever⁴ and Gschwend *et al.*⁵, meanwhile, have pointed out that bromoform is released by temperate macroalgae. Macroalgae, however, are sparsely distributed in the Arctic. Oltmans *et al.*³ proposed that ice microalgae might release significant amounts of organic bromine, but there have been no previous measurements to test this conjecture. In our experiments, bottom ice algae (predominantly pennate diatoms) were tested for organohalogen emission during May 1991 near Resolute Bay, Canada ($74^\circ 39.9' \text{ N}$, $94^\circ 55.2' \text{ W}$, about 1 km offshore). The ice algal layer (roughly the bottom 1 cm of annual ice) was scraped off, diluted with 10 parts of sea water from 15 m to reduce potential osmotic shock^{5,6}, and allowed to melt at -1.5°C . Suspensions of ice algae and sea water were dispensed into 300-ml glass reagent bottles and stoppered, leaving no head space to which volatile compounds might be lost. Incubations were initiated within 30 min of sample collection and normally lasted 3–4 h, centred around local solar noon.

In basic experiments a control bottle containing the same diluent sea water, but no ice algae, was 'incubated' in parallel. In other experiments controls consisted of suspensions held in the dark or with a metabolic inhibitor added. All bottles were stirred and incubated at an irradiance ($\sim 30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) that would saturate but not inhibit photosynthesis⁶. The temperature was maintained at $-1.5^\circ \text{C} \pm 0.2^\circ \text{C}$ by circulating surface sea water (at about -1.7°C) around the bottles.

We collected subsamples of control and experimental treatments for organohalogen analysis before and after incubation to determine net release rates. Aliquots from each treatment

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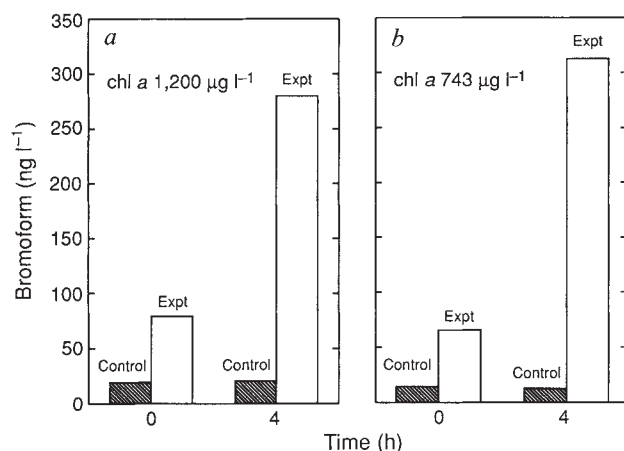


FIG. 1 Results from two representative experiments in which ice algal production of bromoform was measured. Results from the control bottle, containing diluent sea water only, are marked 'Control'. Results from the experimental bottle containing an ice algal cell suspension in the same diluent sea water are marked 'Expt'. Bromoform concentrations are expressed as mass per volume of sea water ($\text{ng CHBr}_3 \text{ l}^{-1}$).

were immediately sparged with purified nitrogen ($100\text{--}200 \text{ ml min}^{-1}$) for 30 min at ambient temperature in a fritted glass sparge vessel. Purged gases were trapped with Tenax TA polymer absorbent. Two single absorbent-filled tubes were used sequentially to determine collection efficiency. The results given here have been corrected for blank levels, sparging efficiency and Tenax collection efficiency, all of which were determined in the field with actual samples. The Tenax tubes were tightly capped, returned to the shore-based laboratory and analysed within two days by a slightly modified analytical procedure⁷. Air samples were collected on identical Tenax tubes, using a battery-operated pump, and analysed in the same manner. Full details will be reported elsewhere.

Experiments with seawater controls showed virtually no change in bromoform concentration during incubation, but the concentration for the algal suspensions consistently increased significantly (Fig. 1). Slightly elevated initial bromoform concentrations in the algal suspensions relative to the control were probably due to release of intracellular material or of bromoform trapped in the ice matrix. Normalized to chlorophyll *a* (chl *a*) concentration, this gives release rates of 42 and 81 $\mu\text{g CHBr}_3 (\text{g chl } a)^{-1} \text{ h}^{-1}$ for Fig. 1a and b, respectively. From a total of 20 similar experiments we measured a range of release rates of 30–330 $\mu\text{g CHBr}_3 (\text{g chl } a)^{-1} \text{ h}^{-1}$. The mean value was $93 \pm 71 \mu\text{g CHBr}_3 (\text{g chl } a)^{-1} \text{ h}^{-1}$ and the median 83 $\mu\text{g CHBr}_3 (\text{g chl } a)^{-1} \text{ h}^{-1}$. Experiments with metabolic inhibitors added to con-

TABLE 1 Estimated global emission of organobromine gases

Source	Emission (10^9 g yr^{-1})
Arctic ice algae (this study)	4.7–70
Antarctic ice algae (this study)	5.3–80
Macroalgae ⁵	4–40
Industrial ⁵	160*

Results are from our work and ref. 5.

* Incorporates methyl bromide fumigant, ethylene dibromide fumigant or automotive emission, Halon flame suppressants and halomethane release (including bromoform) from water chlorination.

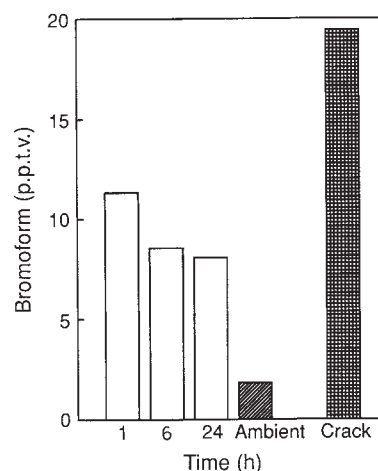
trol suspensions showed that bromoform production was unaffected by a prokaryotic inhibitor, but was essentially halted by the addition of eukaryotic or photosynthetic inhibitors, thus confirming that algae, not bacteria, were responsible for the bromoform production.

Substantial release of bromoform from sea water to the atmosphere was evident in 'flux chambers' placed over freshly drilled holes through the sea ice and over tidal cracks (Fig. 2). The chambers were opaque polyethylene boxes (0.07 m^3) placed directly on the ice over two holes (23 cm diameter) after removing the snow. Their edges were sealed with snow and freezing water. Air was withdrawn through Tenax tubes pushed through a stoppered hole.

High initial bromoform concentrations were measured after 1 h of exposure in a flux chamber (Fig. 2). The levels decayed away probably because of leakage from the box, loss to the walls and gradual refreezing of the holes. Even after 24 h, however, bromoform concentrations inside the chamber were higher than outside air sampled at round the same time. Bromoform concentrations were also elevated in air over a 20–30-cm-wide tidal crack (Fig. 2), and a concentration of 44 parts per 10^{12} by volume (p.p.t.v.) was recorded from over our camp hydrohole. Our results show that bromoform does indeed vent to the atmosphere through openings in the ice. More work is needed to quantify this flux.

Ambient air samples were collected for 3–4 h around solar noon on 11 separate days in May. Bromoform concentrations in air ranged from 1.8 to 3.4 p.p.t.v. with a mean of 2.6 ± 0.6 p.p.t.v. Can the atmospheric concentrations be explained by our observations of bromoform release by ice algae? The chemically perturbed (ozone-depleted) surface inversion layer in the arctic spring is generally less than 500 m thick, and may be as little as 100 m (ref. 8). Taking a layer thickness of 400 m and the above mean atmospheric concentration gives a column amount of $10.7 \mu\text{g CHBr}_3 \text{ m}^{-2}$, if we assume no concentration gradient up to the inversion top and zero concentration above.

FIG. 2 Series of measurements of bromoform in air inside a box placed over two holes drilled through the sea ice. The time is hours from drilling the holes and installing the flux chamber. The shaded bar is the ambient air concentration determined at a distance from the hole on the same day. The cross-hatched bar is the concentration in air above a small tidal crack. Bromoform is expressed as a volumetric mixing ratio (parts per 10^{12} by volume, p.p.t.v.).



Our mean measured bromoform emission rate is equivalent to $2.7 \mu\text{g (g C)}^{-1} \text{ h}^{-1}$, assuming a carbon to chlorophyll conversion of 35 (refs 9, 10). Daylight was continuous but variable during our study, and daily bromoform production would be $32 \mu\text{g (g C)}^{-1} \text{ d}^{-1}$ with only a 12-h photoperiod. In short-term dark incubations, bromoform release was observed, but at reduced rates, so bromoform production may continue in the dark for several hours on stored reserves. Ice algal blooms last 60–90 days at high latitude and biomass levels are typically 10–100 mg chlorophyll m^{-2} (or $0.35\text{--}3.5 \text{ g C m}^{-2}$) in landfast annual ice with production rates of $5\text{--}23 \text{ g C m}^{-2} \text{ yr}^{-1}$ (refs 10, 11). Therefore, an ice algal biomass of $0.35\text{--}3.5 \text{ g C m}^{-2}$ should produce $11\text{--}110 \mu\text{g CHBr}_3 \text{ m}^{-2} \text{ d}^{-1}$.

Periods between surface mixing events are of the order of 1 week (ref. 8). We assume that bromoform accumulates in the surface inversion for 5 days. Over this period release of bromoform to sea water would be $56\text{--}560 \mu\text{g m}^{-2}$. The bromoform column burden calculated above could, therefore, be attained in 5 days if 1.9–19% of the ice algal bromoform was vented to the atmosphere. The actual fluxes from the ice-covered ocean to the atmosphere are not yet known. We have also neglected the atmospheric decomposition rate of bromoform. The lifetime of bromoform in the lower arctic atmosphere in spring may be of the order of weeks¹, or only days or less⁷. Conversely, bromoform could build up under the ice, increasing the source term. Until these issues are resolved, more accurate estimates cannot be made.

Over a year, we presume that most of the bromoform released to surface sea water by marine plants eventually makes its way to the atmosphere, even if not until after the ice breaks up. We used the same approach and basic assumptions as Gschwend *et al.*⁵, who estimated global emissions from measured release rates to sea water of various bromoalkanes from several red, brown and blue-green macrophytes, to estimate global emissions from polar ice microalgae (Table 1). Our annual estimates are based on a mean daily bromoform emission rate for a (conservative) 12-h photoperiod, the typical range for ice algal biomass, the bloom duration and the average extents of annual ice in the Arctic ($7 \times 10^6 \text{ km}^2$) and in the Antarctic ($16 \times 10^6 \text{ km}^2$). Most of the sea ice in the Antarctic is pack ice, which has a lower ice algal biomass than land-fast ice¹¹. We assume mean biomass levels in the Antarctic of $5\text{--}50 \text{ mg chl } a \text{ m}^{-2}$, half that of the Arctic.

Gschwend *et al.*⁵ presented global estimates of organohalogen production for macrophyte and anthropogenic sources, which are of the same order as those for ice algae (Table 1). Their estimates are the sums of several brominated gases; the values for bromoform alone would be less. Furthermore, the macrophyte growing season was taken to be 365 days, which is an overestimate. Our Table should not, however, be considered to indicate an apportionment of the atmospheric burden of organic bromine, as the different chemical species have very different lifetimes. Instead it illustrates the magnitude of the bromoform production by ice algae. □

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Oil and combustion-product contamination of the Gulf marine environment following the war

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FOLLOWING the Gulf war, controversy and speculation have surrounded the extent to which the massive spillage of petroleum and the burning of oil wells in Kuwait have damaged marine ecosystems in the region^{1–5}. We report here the results of a rapid assessment survey of hydrocarbon contamination undertaken in the coastal marine environment from Kuwait to Oman during mid-1991. Our results show that severe oil pollution was restricted primarily to the Saudi Arabian coastline within ~400 km from the spillages, and that during the four months following the conflict and preceding our survey, the spilled oil had extensively degraded. Surprisingly, concentrations of petroleum hydrocarbons in sediments and bivalve molluscs from Bahrain in June 1991 were lower than those recorded from our pre-war (1983–86) surveys at the same site, probably as a result of decreased tanker traffic and associated deballasting during and after the conflict. As for carcinogenic polycyclic aromatic hydrocarbons produced during burning of the oil wells, we found that concentrations in sediments from even the most heavily contaminated sites were relatively low, and comparable to levels reported for the Baltic Sea⁶, coastal locations of the northeastern United States⁷ and United Kingdom estuaries⁸.

Estimates of the quantities of oil directly released into the Gulf during the war from, or near, the Sea Island terminal in Kuwait have ranged from 0.5 to 8 million barrels^{3,4}. Furthermore, from the best available data^{2,4} it can be estimated that over the ~250 days during which the wells were burning, some 500 million barrels (67 million tonnes) of oil were emitted or ignited, releasing oil aerosols, soot, toxic combustion products and gases for atmospheric transport and subsequent deposition. Even if only a few per cent of those emissions fell out in the coastal marine environment, they would far surpass the amounts of crude oil that were spilled. To our knowledge, no scientific reports have appeared that quantitatively describe the extent of hydrocarbon contamination in Gulf waters, although some address potential environmental and climate effects from the pollution^{1,2,4,9–11}. Following the war we collected coastal samples from throughout the Arabian coastline of the Gulf. Aerial and satellite observations⁴ supported physical oceanographic data on prevailing currents¹² and identified this area to be initially affected and at most risk from the spilled oil.

During three visits to the Gulf from June to October 1991 we collected 14 samples of nearshore sediments, nine composite samples each comprising 20–30 specimens of a single species of bivalve mollusc (*Meretrix meretrix*, *Tapes sulcarius* and *Trachycardium lacunosum* from Saudi Arabia; *Pinctada margaritifera* from Bahrain and United Arab Emirates; *Spondilus* sp. from Bahrain; *Saccostrea cucullata* from Oman) and 17 samples of fish (*Arius thalassinus* from Kuwait; *Lethrinus nebulosus* from Saudi Arabia; *Epinephelus tauvina* from Bahrain and Oman; *Epinephelus jayakari* from Bahrain; *Acanthopagrus bifasciatus* from Bahrain; and *Epinephelus suillus* from United Arab Emirates). In Kuwait, access to the entire coastline and nearshore waters was forbidden owing to mines and unexploded ordnance. We were, however, able to travel by helicopter to Qaruh Island (station 1, Fig. 1) to collect sediment samples and, in addition, to obtain freshly caught fish from near Kuwait City within ~20 km of the spills. For the other countries visited, sample locations that had been visited in previous surveys^{13,14}

were preferentially selected. Sampling and sample preparation procedures were done according to internationally recognized guidelines^{15,16}.

Frozen samples were returned to the laboratory, freeze-dried and analysed according to the methods used in our previous studies^{13,16}. Sediments were solvent-extracted and residual sulphur was removed. Biota samples were solvent-extracted and saponified. The sediment extracts and the non-saponifiable lipids from the biota were then fractionated by column chromatography on silica gel/alumina to provide 'aliphatic' and 'aromatic' fractions. The fluorescence intensities were then compared with Kuwait crude oil (the reference oil selected as an analytical standard by the Regional Organization for the Protection of the Marine Environment (ROPME)). Additional syn-

chronous excitation and emission spectra were also obtained to characterize the residues¹⁶. Fractions were then analysed by capillary gas chromatography with flame ionization detection (GC-FID)¹⁶. Gas chromatography-mass spectrometry (GC-MS) was used for confirmatory analyses¹⁶.

During a helicopter flight along the southern Kuwait coast (June 1991), several small slicks were observed, especially in areas of the terminals that had suffered war damage. Nevertheless, except for some localized oil patches which came ashore, the beaches appeared from the air to be relatively clean. Tar-ball surveys on Qaruh island (station 1, Fig. 1) revealed levels ($\sim 380 \text{ g m}^{-1}$ of beach) similar to those recorded during pre-war surveys in Kuwait¹⁷ and other Gulf States¹³.

Concentrations of oil hydrocarbons (as determined by the relative fluorescence of combined aliphatic and aromatic fractions compared with Kuwait Crude oil¹⁶) are shown in Fig. 1. Sediment and bivalve analyses indicate that the main oil contamination has been restricted to the shoreline of Saudi Arabia within $\sim 400 \text{ km}$ of the release sites. Stations outside this area (including Muscat on the Indian Ocean shoreline of Oman; Fig. 1, station 14) have similar comparatively low levels of contamination. This trend of distribution is most clearly delineated in sediment samples but is strongly supported by the bivalve mollusc data which also show an overall decrease with distance from the area of impact. Although concentrations in fish species seem slightly elevated in the most northerly samples, their relative degree of contamination is lower than that observed for the bivalve molluscs. This may be because the fish can avoid oil spills and/or because they rapidly depurate or metabolize oil. Although sampling was conducted throughout a four-month period, satellite observations⁴ showed that extensive dispersion of the slick occurred rapidly (and well before our sampling commenced). In addition, it is apparent from Fig. 1 that the highest concentrations were in samples obtained from Saudi Arabia in the middle of the collection period. For these reasons we believe that the gradients described are not substantially influenced by temporal differences in our sampling and truly reflect the spatial trends in contamination.

If the area outside the directly affected locations of Kuwait and Saudi Arabia is considered, the relative degree of oil pollution from the war can be evaluated by comparison with our data obtained in previous years (Fig. 2). For example, a sampling location off Askar on the eastern coast of Bahrain (station 10, Fig. 1) was revisited to obtain sediment and oyster samples. Compared with our earlier samples, there is an apparent decrease in hydrocarbon contamination. 'Normal' oil pollution inputs into the northern Gulf from oil production and transport activities are on average ~ 2 million barrels per year⁴. This represents roughly half of the quantity spilled, essentially from a point source, during the conflict. Given the considerable reduction in these activities between late 1990 and 1991, the low petroleum hydrocarbon levels that we recorded have probably resulted from reduced chronic inputs during that period.

A more detailed appraisal of the situation is available from results of our GC-FID analyses of sediment extracts (Fig. 3). The same pattern of severe contamination at locations along the northwestern coast of Saudi Arabia is revealed, although greater spatial variability is indicated, probably because of localized topographical and depositional coastal characteristics.

To investigate the changes that occur when petroleum is spilled into the marine environment, it is essential to appreciate that oil is a composite of thousands of individual compounds with differing physico-chemical properties. These weather at differing rates once released into the sea. In its original (undegraded) state the main individual components of the oil (as determined by GC) are the *n*-alkanes. Some of these compounds (*n*-C₁₄ to *n*-C₃₄) are frequently used to identify 'fresh' inputs of petroleum⁸. During the first stages of weathering (and within days of the spillage) volatilization depletes the lower-molecular-weight *n*-alkanes (roughly, those below *n*-C₁₄)

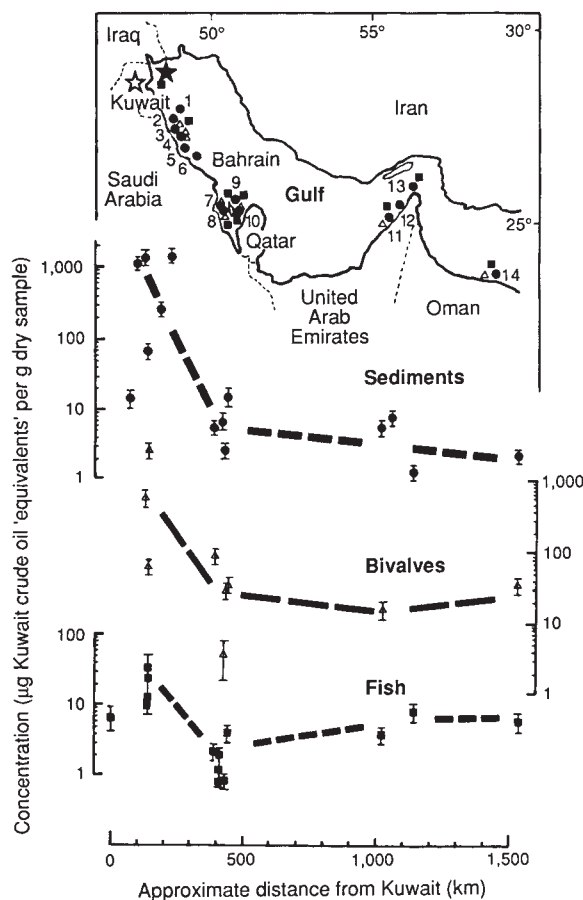


FIG. 1 Concentrations of petroleum hydrocarbons (as estimated by fluorescence comparison with Kuwait crude oil) in individual samples of subtidal surface (0–2 cm) sediments, bivalve molluscs (soft parts) and fish (muscle). The approximate locations of the oil spills and burning wells are depicted by a filled and an open star respectively (see text for further details). Locations for individual sampling stations are indicated by circles, triangles and squares for sediments, bivalve molluscs and fish respectively. The notation for samples in the map is repeated for the graphs. Sample numbers refer to the sediment sampling stations shown in Fig. 3. Samples from the Bahrain area (near stations 8 to 10) and Kuwait (station 1) were collected in mid- to late June 1991, those from Saudi Arabia (stations 2 to 7) in mid-August 1991 and those from the United Arab Emirates and Oman (stations 11 to 14) in late September or early October 1991. Bivalve mollusc samples are composites (comprising 20–30 individuals) of single species available at the various locations. Fish concentrations represent analyses of the muscle from individual specimens obtained. For each individual point an error bar is included to reflect analytical variability (as determined by triplicate analyses of a single homogenized sample). Bold broken lines indicate trends in 'oil' contamination. The 'distance from Kuwait' scale, which is used to delineate the sample locations, gives the approximate relative distances from the pollution sources using a coastal (rather than a direct) trajectory.

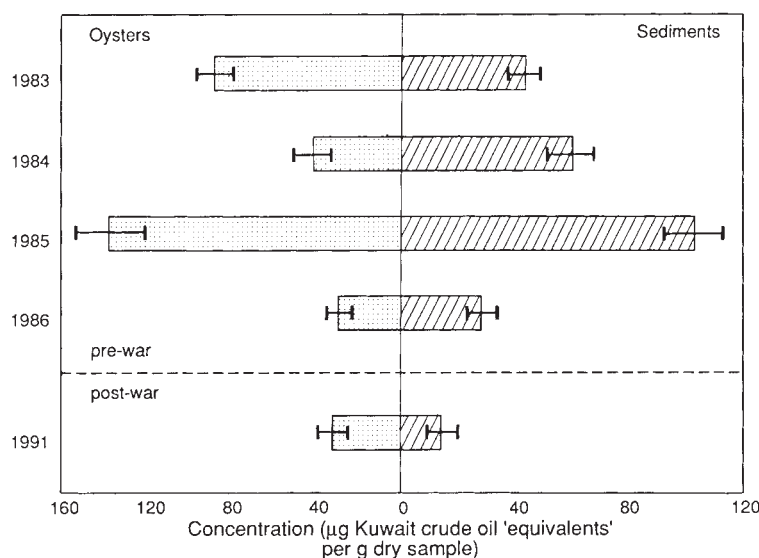


FIG. 2 Concentrations of petroleum hydrocarbons in a composite ($n=20$ to 30) sample of pearl oysters (*Pinctada margaritifera*) and sub-tidal surface ($0-2$ cm) sediments sampled from a single site at Askar on the eastern coast of Bahrain (station 10, Fig. 1) in consecutive years from 1983 to 1986 (ref. 13; S. Fowler, unpublished results) together with post-war results. Concentrations are expressed as micrograms Kuwait crude oil equivalents per gram dry weight and were determined by fluorescence analyses. Error bars indicate the analytical variability as determined from triplicate analyses of a single homogenized sample.

together with volatile aromatic compounds. Dissolution and biodegradation also act to reduce rapidly the concentrations of these components¹⁸. During subsequent weeks and months, heterotrophic microbes further (and selectively) reduce n -alkane concentrations and, together with photooxidation, aromatic hydrocarbons are also degraded. Coinciding with removal of these components is an increase in unresolved components

within the gas chromatograms¹⁸ (often referred to as the unresolved complex mixture). The composition of this mixture is only poorly understood¹⁹, but it is frequently used as a relative measure of chronic/degraded petroleum contamination⁸. Although sampling took place within a few months of the main oil spill (which occurred in late January 1991), the very low ratios (<0.2) of resolved to unresolved hydrocarbons indicate that within this period most of the spilled oil in the sediments had substantially degraded into an unresolved complex mixture⁸. The highest values of this ratio (which indicates 'most recent' inputs of oil) were recorded off Bahrain (stations 7 and 8; Fig. 1). Analyses revealed, however, that fuel oil rather than Kuwait crude oil was responsible for this localized contamination. In addition, the relatively small contributions of n -alkanes ($n\text{-C}_{14}$ to $n\text{-C}_{34}$) to the total hydrocarbons confirm that most of the oil spilled in Kuwait had substantially degraded. The high value at Ras Al Ghar (station 6, Fig. 1) might, however, have resulted from deposition of a 'protected' and remobilized oil pocket originating from the Kuwaiti spills.

Pollution from polycyclic aromatic hydrocarbons (PAH) will have arisen from both the oil spills and burning of the wells. The low-molecular-weight (≤ 3 aromatic ring) compounds together with their numerous alkylated homologues are typical constituents of crude oils^{8,18}. GC analyses confirmed that these were the main resolved aromatic components. It is apparent from Fig. 3 that when summed, the resolved aromatic compounds generally covary with the oil contamination (as indicated by the total hydrocarbons). PAH formed during high-temperature combustion (as might be expected in the burning of oil wells) are generally larger molecular weight (≥ 4 aromatic ring) parental (non-alkylated) compounds, many of which are carcinogenic. Pyrene is a principal constituent of the generally uniform assemblage of PAH associated with combustion^{7,8}. The distribution of this compound (as an indicator of combustion-derived PAH) is shown in Fig. 3. Concentrations in the Gulf sediments ($3-450$ ng per g dry sediment; Fig. 3) are well within the range of those reported for other coastlines, such as Buzzards Bay and New York Bight, USA ($7-1,300$ ng per g dry weight)⁷ and estuaries within the United Kingdom ($60-1,510$ ng per g dry weight)⁸. Indeed the median value for the Gulf sediments (12.5 ng per g dry weight) is lower than that for a survey of similar scope and sediment type (mostly sandy) in the Southern Baltic sea⁶ (72.5 ng per g dry weight; range $1.7-150$ ng per g dry weight). PAH emanating from the burning oil wells do not seem to have broadly contaminated the coastal areas investigated. □

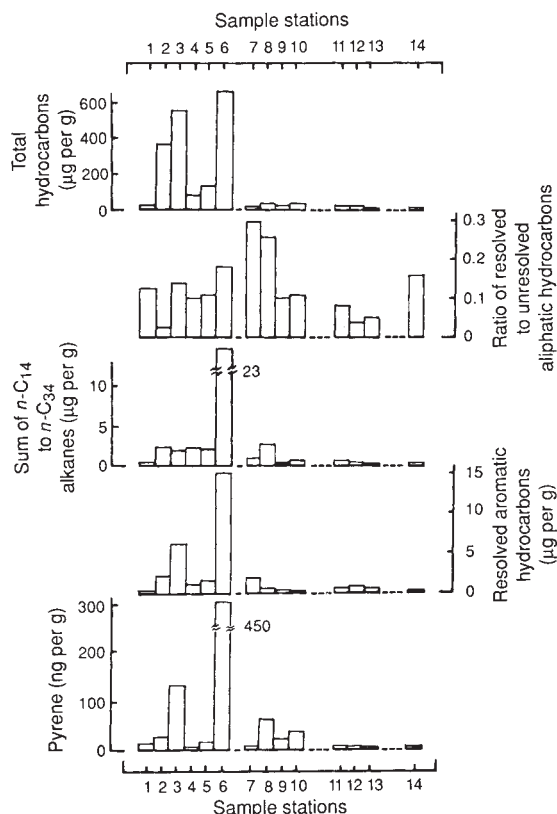


FIG. 3 Concentrations of hydrocarbon compounds together with relevant ratios (see text for explanation) obtained from gas chromatographic analyses of the sediments. Locations of the sample stations are shown in Fig. 1. Analytical precisions for the individual parameters (as determined by triplicate analyses of a single homogenized sample) were: total hydrocarbons, $\pm 13\%$; resolved aliphatic hydrocarbons, $\pm 5\%$; unresolved aliphatic hydrocarbons, $\pm 13\%$; the sum of $n\text{-C}_{14}$ to $n\text{-C}_{34}$ alkanes, $\pm 6\%$; resolved aromatic hydrocarbons, $\pm 12\%$; and pyrene, $\pm 10\%$.

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Enhanced ventilation of the North Atlantic subtropical gyre thermocline during the last glaciation

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THE ocean's intermediate^{1–6} and deep^{7,8} circulation are both known to have differed during the last glaciation from those of today, but little is known about the history of the subtropical gyres. Variations in gyre processes should be closely linked to variations in global climate, as gyre circulation is driven by air–sea interactions and reflects the climate at the ocean surface. The gyres are also a significant reservoir of carbon and nutrients, so that gyre processes affect the distribution of carbon and nutrients in the oceans and thus atmospheric CO₂. Here we use measurements of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in foraminifera from the Bahamas to produce a detailed reconstruction of nutrient and temperature profiles in the thermocline during the last glaciation. The thermocline of the glacial North Atlantic subtropical gyre lacked the oxygen minimum that is characteristic of the modern ocean, and was depleted in nutrients, indicating greater, more uniform thermocline ventilation at that time. Thus not only intermediate waters^{1–5} but the entire upper water column within the glacial North Atlantic was depleted of nutrients. Moreover, glacial thermocline waters were cooler, had a steeper temperature gradient and a shallower base. Both the ventilation and the thermal structure of the glacial thermocline are consistent with what is known of the glacial climate^{9–14}.

The subtropical gyre of the western North Atlantic is characterized by a steep vertical temperature gradient (the main thermocline) that extends from the base of the mixed layer (~150 m) to uniformly cool deep waters (~1,200 m). Within the thermocline, vertical gradients of water properties reflect lateral flow of water along isopycnal surfaces^{15,16}. Wind-driven Ekman

transport and buoyancy (density) flux drive water into the thermocline during late winter when the mixed layer reaches its maximum depth. Water then flows along isopycnals to greater depths at lower latitudes, conserving potential vorticity along the way. In this ventilated region of the thermocline, the fluxes and trajectories of water (and thus the thermocline's vertical structure), depend largely on the climatic conditions at the region where isopycnals outcrop during late winter. Circulation, together with *in situ* reactions with settling particles, controls the vertical distribution of nutrients. Photosynthesizing marine plants in the surface mixed-layer produce oxygen and deplete surface waters of nutrients and ^{12}C , while the decomposition of settling organic matter at depth consumes oxygen and releases nutrients and ^{12}C back into the water. As a result, the concentrations of nutrients and ΣCO_2 increase with depth, whereas the concentration of dissolved oxygen and the $\delta^{13}\text{C}$ of ΣCO_2 decrease^{17,18}.

Today, a maximum in phosphate and minima in dissolved oxygen and $\delta^{13}\text{C}$ of ΣCO_2 occur in lower thermocline waters (potential density anomaly ~27.2–27.5 kg m⁻³) which lie between the oxygen-rich upper layers of the thermocline and the upper component of the North Atlantic Deep Water^{19,20}. The oxygen concentration primarily reflects how well ventilated the waters are. Waters are saturated with oxygen at the ocean surface, but when they are subducted into the thermocline biological consumption outpaces supply by advection or diffusion, and the oxygen concentration falls. The well-ventilated region of the thermocline corresponds to isopycnals that outcrop in the subtropical gyre, where waters are driven directly into the thermocline by Ekman pumping and buoyancy flux²¹. They circulate rapidly around the gyre and are frequently ventilated as indicated by high tritium concentrations and young tritium-³He ages^{23–27}. By contrast, waters associated with the oxygen minimum are poorly ventilated. They correspond to isopycnals that outcrop north of the line of zero wind-stress curl, where Ekman suction occurs—this does not contribute to thermocline ventilation^{21–28}. These waters also circulate about the subpolar gyre before communicating with the subtropical gyre, depleting oxygen and accumulating nutrients^{15,21,54}. The volume of poorly ventilated and low-oxygen waters increases along the southeastern margin of the subtropical gyre^{24–28}, reflecting degradation of organic matter beneath the upwelling zone off Africa, and to some extent the anticyclonic trajectory of water subducted from the gyre surface²¹. These low-oxygen waters are a potential contributor to the oxygen-minimum waters of the western North Atlantic subtropical gyre²⁹.

The margins of Little and Great Bahama Banks, which bound the eastern end of Northwest Providence Channel, are among the few topographic features in the western North Atlantic that intersect subtropical gyre thermocline waters away from the effects of continental margins. The channel is part of a seaway that connects the western North Atlantic with the Florida Straits. Today, temperature–salinity, oxygen–density and temperature–depth relationships of thermocline and underlying waters within the seaway are characteristic of the Sargasso Sea and North Atlantic Deep Water^{30–32}. An underwater sediment spur isolates the waters below ~450 m in Northwest Providence Channel from those in the Florida Straits, so the source of water below this depth must be the western subtropical gyre³¹.

A suite of box, gravity and piston cores was recovered from a limited area along the bank margins (25.9–26.3° N, 77.6–78.2° W) with a depth distribution (425–1,534 m) that spans the main thermocline and extends into upper North Atlantic Deep Water. Each core was visually inspected and sampled at regular intervals, and the percentage of aragonite relative to total aragonite and calcite in bulk sediment was determined by X-ray diffraction (Fig. 1). The $\delta^{18}\text{O}$ values of planktonic foraminifera (*Globogerinoides sacculifer*, 300–350 μm or *G. ruber*, 212–250 μm) from the tops (late Holocene) and glacial-aged sections of each core were measured by mass spectrometry. High

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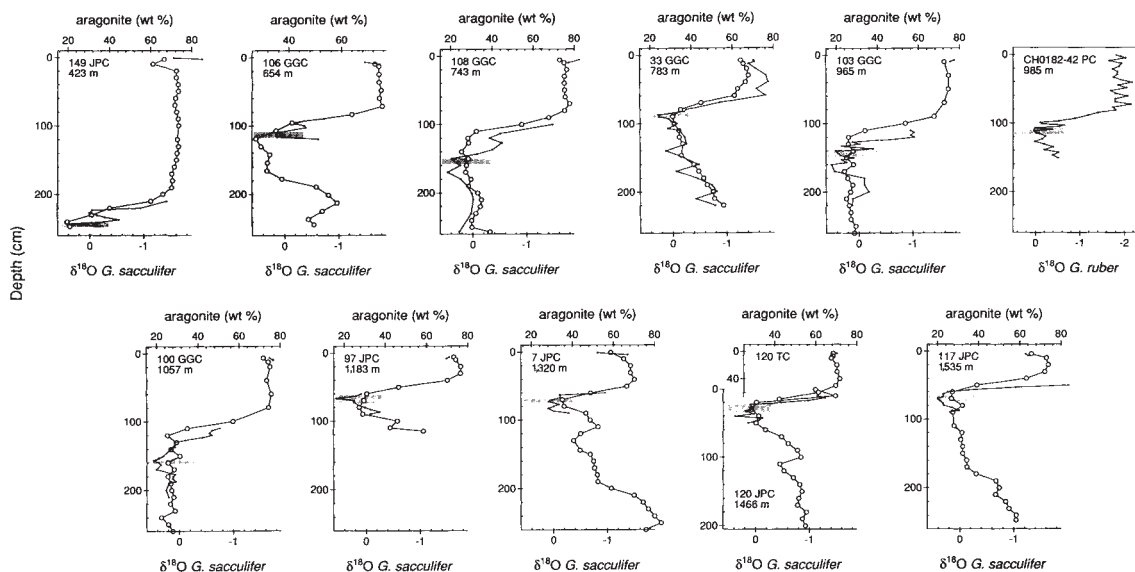


FIG. 1 Aragonite content (○) and $\delta^{18}\text{O}$ of planktonic foraminifera (●) plotted against depth in the upper 260 cm of piston and gravity cores recovered from the margins of Little and Great Bahama Banks. Core numbers and water depths are given on each panel. Aragonite content and $\delta^{18}\text{O}$ were measured with precisions of $\pm 1\%$ and $\pm 0.1\%$, respectively, at the Woods Hole Oceanographic Institution. High aragonite and low $\delta^{18}\text{O}$ at the core tops indicate that they are late Holocene in age, whereas low aragonite and high $\delta^{18}\text{O}$ indicate glacial-aged sediments^{33,34}. Benthic foraminifera were picked from core tops and sediments deposited during the last glacial maximum (LGM, indicated by stippling). LGM to core-top sedimentation rates

aragonite and low $\delta^{18}\text{O}$ at the core tops indicate that they are late Holocene in age, whereas low aragonite and high $\delta^{18}\text{O}$ identify the glacial-aged sediments^{33,34}. Isotope ratios of benthic foraminifera *Planulina foveolata*, *P. ariminensis* and *P. wuellerstorfi* ($>250\ \mu\text{m}$) from late-Holocene and last-glacial-maximum (LGM) sediments were measured by mass spectrometry. Gradients of the isotopic compositions of *Planulina* species are believed to represent gradients of the $\delta^{13}\text{C}$ of ΣCO_2 and the

range from ~ 3 to $13.5\ \text{cm kyr}^{-1}$. The aragonite profile of core 120 JPC indicated that some Holocene sediments were missing, so it has been spliced together with its trigger weight core (120T). Similar LGM to late Holocene ranges of aragonite content and $\delta^{18}\text{O}$ in the cores suggest no significant disturbance by bioturbation or coring processes. Core 7 JPC may be an exception, as it has a low sedimentation rate and the smallest ranges of aragonite content and $\delta^{18}\text{O}$ (see benthic values in Figs 2 and 3). Core 149 JPC barely penetrated the glacial sediments. Thus, although the aragonite content and $\delta^{18}\text{O}$ of *G. sacculifer* at its base are consistent with a LGM age, we cannot be certain on this basis alone.

FIG. 2 Vertical profiles of (a) seawater oxygen and phosphate concentrations near the Bahamas⁴⁴, and (b) two estimates of the $\delta^{13}\text{C}$ of ΣCO_2 in sea water (—, offset by 0.2‰ to compensate for foraminiferal vital effects) and the average $\delta^{13}\text{C}$ of late Holocene and glacial benthic foraminifera (squares, *P. foveolata*; circles, *P. ariminensis*; triangles, *P. wuellerstorfi*). Open symbols indicate Holocene, filled symbols indicate glacial). $\delta^{13}\text{C}$ values of glacial foraminifera do not possess a local minimum and are more positive than values of late Holocene foraminifera, indicating that during the last glacial maximum there was no oxygen minimum and waters were depleted in nutrients. $\delta^{13}\text{C}$ of ΣCO_2 estimates are based on along-isopycnal differences in phosphate and oxygen between the GEOSECS II station¹⁷ and the Bahamas, given the water $\delta^{13}\text{C}$ and ΣCO_2 at GEOSECS II¹⁸ and assuming phosphate and carbon are released into the water and oxygen is consumed in Redfield proportions during decay of organic matter with $\delta^{13}\text{C}$ of -18.1% . Benthic foraminiferal isotopic compositions were measured at the Lamont-Doherty Geological Observatory (average standard deviations of 15 duplicate $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ analyses were 0.05 and 0.02‰, respectively). Bars on foraminiferal values indicate the standard error of averaged values and question marks indicate data for which we suspect slight downslope creep of foraminifera shells. Assuming average core-top foraminifera are at least 100 years old, their $\delta^{13}\text{C}$ does not reflect the recent input into the oceans of isotopically light CO_2 from fossil fuels, whereas the $\delta^{13}\text{C}$ of rapidly ventilated thermocline waters to some extent does³⁶. Sea level during the LGM is taken to be 120 m lower than at present³⁵.

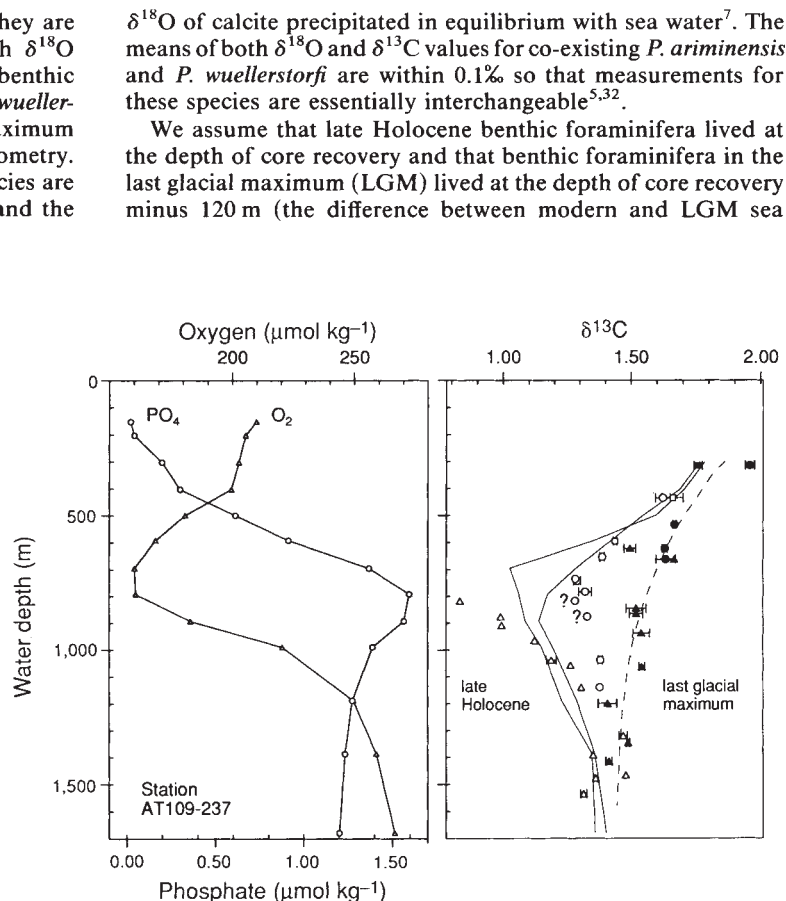
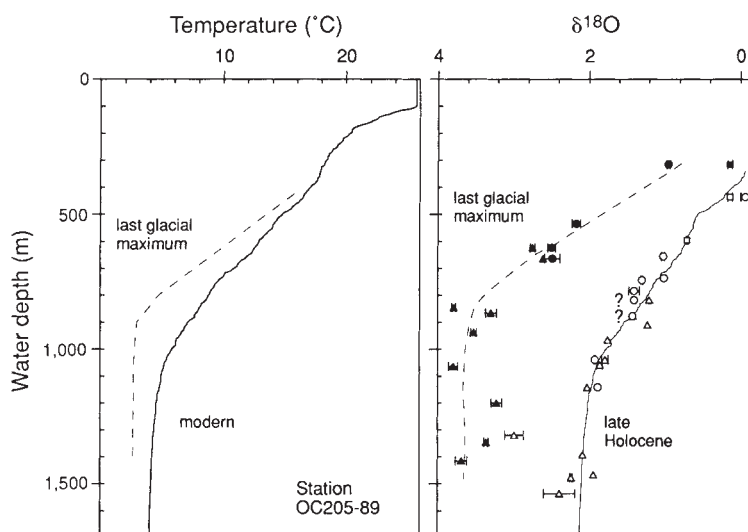


FIG. 3 Vertical profiles of (a) water temperature near the Bahamas³² today (—) and estimated for the last glacial maximum (---), and (b) the $\delta^{18}\text{O}$ of equilibrium calcite (—, offset by -0.9% to compensate for foraminiferal vital effects) and the average $\delta^{18}\text{O}$ of late Holocene and glacial benthic foraminifera (symbols are as in Fig. 2). A steep gradient of water temperature with depth characterizes the modern thermocline and is reflected in gradients of equilibrium and foraminiferal $\delta^{18}\text{O}$. During the LGM (---), foraminiferal $\delta^{18}\text{O}$ values were more positive, their gradient was steeper, and the inflection in this gradient that marks the thermocline base was shallower than during the late Holocene. These data indicate that the temperature gradient was steeper and waters were cooler during the last glaciation. Glacial water temperatures were estimated from the change in foraminiferal $\delta^{18}\text{O}$ (assuming the bathymetric gradient of $\delta^{18}\text{O}_{\text{water}}$ remained constant through time and the $\delta^{18}\text{O}$ of mean sea water was 1.2% greater during the glaciation³⁵). During the LGM, sea level is taken to be 120 m lower than at present³⁵. Equilibrium calcite $\delta^{18}\text{O}$ was calculated from modern water temperatures, $\delta^{18}\text{O}_{\text{water}}$ -salinity relations for the Sargasso Sea and North Atlantic Deep Water⁴⁵, and the palaeotemperature relationship of Erez and Luz⁴⁶.



levels³⁵). We plotted the average $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values for these foraminifera against water depth to construct vertical profiles. The Holocene profile of foraminiferal $\delta^{13}\text{C}$ displays minimum values at about the same depth ($\sim 700\text{--}900\text{ m}$) as the hydrographic maximum in phosphate and the minima in dissolved oxygen and $\delta^{13}\text{C}$ of ΣCO_2 (Fig. 2). Foraminiferal $\delta^{18}\text{O}$ increases from near the sea surface to $\sim 1,100\text{ m}$ depth and is more uniform below, reflecting the temperature gradient of the main thermocline and the upper North Atlantic Deep Waters which underlie the base of the thermocline (Fig. 3). These close correspondences between gradients of modern hydrographic properties and Holocene *Planulina* isotopic compositions lend confidence to the use of fossil $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ to reconstruct the glacial thermocline.

Unlike the Holocene values, LGM $\delta^{13}\text{C}$ values do not show a local minimum; rather, they decrease steadily with depth, suggesting that there was no oxygen minimum layer within the glacial thermocline (Fig. 2). Also, the $\delta^{13}\text{C}$ of LGM *Planulina* are at least $0.1\text{--}0.2\%$ higher and as much as 0.6% higher at the depth of the modern ocean $\delta^{13}\text{C}$ minimum, indicating that glacial nutrient concentrations were less than today. If we take -0.93% per $\mu\text{mol per kg}$ as the ratio of changes in $\delta^{13}\text{C}$ to changes in phosphate³⁶ and $\sim 0.3\%$ as the glacial to recent shift in mean ocean $\delta^{13}\text{C}$ (ref. 8), we can estimate that glacial phosphate concentrations within the thermocline were $\sim 0.4\text{--}0.9\text{ }\mu\text{mol kg}^{-1}$ less than today. Under these assumptions, the deepest waters studied here have a glacial phosphate content of $\sim 0.8\text{ }\mu\text{mol kg}^{-1}$, similar to the value inferred³ from glacial Cd/Ca in a Caribbean core, KNR 65 station 5 core 5PG, which monitors similar glacial waters. The consistency between $\delta^{13}\text{C}$ (affected by gas exchange) and Cd/Ca (not affected by gas exchange) suggests that greater glacial $\delta^{13}\text{C}$ values are not simply an artefact of greater gas exchange and carbon isotope equilibrium between the atmosphere and the surface waters of the North Atlantic during the glaciation.

Late Holocene and LGM $\delta^{18}\text{O}$ profiles extend our preliminary consideration of the thermocline's physical structure³⁷, indicating that it differed between the LGM and today in several ways (Fig. 3). The magnitude of the glacial isotopic gradient, $d(\delta^{18}\text{O})/dz$, was greater and the base of the thermocline was shallower by $\sim 100\text{ m}$. Glacial values are 1.4% (shallow) to 2.1% (deep) higher than Holocene values. About 1.2% of this difference reflects a change in the average isotopic composition of the ocean due to the accumulation of glacial ice³⁵, and the remaining $0.2\text{--}0.7\%$ difference results from some combination of a decrease in water temperature and a change in the local bathymetric gradient of seawater $\delta^{18}\text{O}$. Assuming that this

gradient remained constant with time, we estimate that the upper $1,500\text{ m}$ of the water column was cooler by at least $\sim 1\text{ }^\circ\text{C}$ during the LGM and the deepest of these waters were up to $4\text{ }^\circ\text{C}$ cooler. This result provides independent corroboration of observations⁹⁻¹¹ that the mid-latitude North Atlantic surface ocean, the source of thermocline waters, was $\sim 2\text{--}4\text{ }^\circ\text{C}$ cooler in winter during the LGM.

At least two processes could result in lower nutrient concentrations and the absence of the phosphate maximum and oxygen minimum within the glacial thermocline: greater, more uniform ventilation of thermocline waters; and less degradation of organic matter off Africa. It is difficult to reconcile the latter possibility with evidence that productivity off Africa was actually greater during the LGM³⁸. But results from climate models are consistent with the idea that air-sea interactions where thermocline isopycnals outcropped in the glacial North Atlantic resulted in greater ventilation. Glacial westerlies were stronger¹¹⁻¹³, implying greater Ekman pumping and increased ventilation of isopycnals that outcropped within the subtropical gyre. As westerlies flowed along the winter margin of sea ice¹¹⁻¹³, the subtropical gyre boundary was at or only slightly south of the ice margin¹⁴ and all thermocline isopycnals, including those that correspond to the current oxygen-minimum layer, probably outcropped in the region of Ekman downwelling. Buoyancy flux processes also aid in thermocline ventilation³⁹⁻⁴² and were probably enhanced during the LGM. For example, stronger and drier winds¹¹⁻¹³, storm tracks along the ice margin¹²⁻¹⁴, and, locally, sea ice development might all enhance deep winter convection by increasing the water's heat loss to the atmosphere and its salinity. Some of these mechanisms have been generally discussed in regard to the formation of glacial North Atlantic Intermediate Water^{3,8}.

Our data show that not just intermediate-depth waters¹⁻⁵ but the entire upper water column of the North Atlantic were depleted in ^{12}C and nutrients during the LGM. This vertical redistribution of carbon may have influenced the concentration of atmospheric CO_2 (ref. 43). As the thermocline is more depleted in ^{12}C than the underlying intermediate-depth waters, thermocline ^{12}C depletion cannot result from simple mixing with intermediate-depth waters. We suggest that thermocline processes, being intimately linked with surface ocean climate and ocean carbon cycling, are crucial in the ocean's response to and, perhaps, active participation in climate change. Further thermocline reconstructions are necessary to verify that the nutrient depletion observed at the Bahamas was a gyre-wide phenomenon and to sort out the variable response of thermocline structure to local climate-forcing. □

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Optical imaging of epileptiform and functional activity in human cerebral cortex

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OPTICAL imaging of animal somatosensory, olfactory and visual cortices has revealed maps of functional activity^{1–12}. In non-human primates, high-resolution maps of the visual cortex have been obtained using only an intrinsic reflection signal^{3,4,6,13,14}. Although the time course of the signal is slower than membrane potential changes, the maximum optical changes correspond to the maximal neuronal activity^{3,6}. The intrinsic optical signal may represent the flow of ionic currents, oxygen delivery, changes in blood volume, potassium accumulation or glial swelling^{3,4,6,8,15–18}. Here we use similar techniques to obtain maps from human cortex during stimulation-evoked epileptiform afterdischarges and cognitively evoked functional activity. Optical changes increased in magnitude as the intensity and duration of the afterdischarges increased. In areas surrounding the afterdischarge activity, optical changes were in the opposite direction and possibly represent an inhibitory surround. Large optical changes were found in the sensory cortex during tongue movement and in Broca's and Wernicke's language areas during naming exercises. The adaptation of high-resolution optical imaging for use on human cortex provides a new technique for investigation of the organization of the sensory and motor cortices, language, and other cognitive processes.

We measured optical changes in response to bipolar cortical stimulation in five patients undergoing surgery for intractable epilepsy^{19,20}. The feasibility of measuring optical changes during such human cortical stimulation had been proposed previously (B. MacVicar, D.W.H., T. Watson and F. LeBlanc, personal communication). In our stimulation studies, we correlated the optical changes with surface electrical recordings (surface EEG). Figure 1Aa shows the results obtained when the surface EEG and stimulating electrodes (r and s, respectively) were placed just anterior to the region of the motor cortex controlling the

facial musculature. Four successive stimulations were followed by epileptiform afterdischarges of varying intensity and duration (see lower trace of Fig. 1B for surface EEG recordings during stimulations 2 and 4). Long afterdischarges (12–16 s) followed stimulations 1 (6 mA) and 4 (8 mA), whereas stimulations 2 and 3 (6 mA) elicited shorter afterdischarges (<4 s).

The size of the active region and the amplitude of optical changes at specific sites were compared in the four recordings. The optical changes between the stimulating electrodes (site 1, Fig. 1A) and near the recording electrode (site 2) show a graded response to the intensity and duration of the afterdischarges (Fig. 1B). The spatial extent of the epileptiform activity is calculated by comparing a normalized baseline image collected before stimulation (Fig. 1Ab) to images obtained immediately after stimulation. The intensity and spread of the optical changes were less following stimulation 2 (6 mA, <4 s afterdischarges; Fig. 1Ac) than after stimulation 4 (8 mA, 16 s afterdischarges; Fig. 1Ad). The intensity and duration of the optical changes (in response to stimulation above and below the threshold for afterdischarge activity) correlated with the electrical changes.

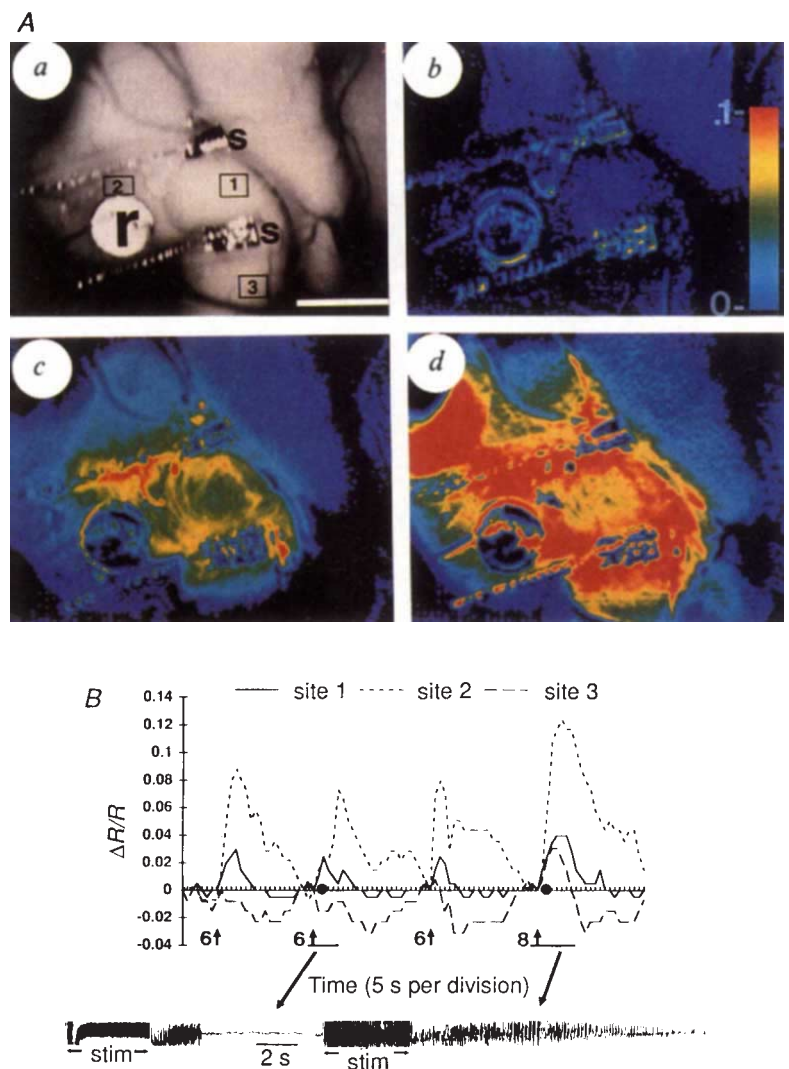
Optical changes associated with afterdischarge activity were observed to shift below the baseline in three patients (black regions in Fig. 1Ac, negative signal in Fig. 1B, site 3). This negative optical signal may represent inhibited neuronal populations (an epileptic inhibitory surround), decreased oxygen delivery, or blood volume shunted to activated regions^{3,8}. In the more intense afterdischarge episodes, the optical change below baseline was delayed (stimulation 4; Fig. 1B).

Stimulation mapping of the cortical surface was also done on awake patients under local anaesthesia to identify the sensory cortex (sites 1 and 2; Fig. 2Aa) and Broca's area (sites 3 and 4; Fig. 2Aa)^{19,20}. We defined Broca's area as the site where speech arrest occurred during cortical stimulation (4–8 mA). Images were collected at rest and while the patient moved the tongue from side to side with the mouth closed. Optical changes during three trials (Fig. 2C) were greatest at cortical sites where stimulation (3 mA) had evoked sensations in the palate (site 1, Fig. 2Aa) and tongue (site 2, Fig. 2Aa) ($n = 2$ subjects). The spatial extent of the optical changes in the sensory cortex is shown by comparing the normalized control image obtained at rest (Fig. 2Ab) to a normalized image obtained during tongue movement (Fig. 2Ac). The largest optical changes occur in the sensory cortex; in other areas the optical changes are in the opposite direction.

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FIG. 1 *Aa*, Exposed cortical surface anterior to the motor cortex region controlling the facial muscles, with one recording (r) and two stimulating (s) electrodes, and three sites (1–3) where optical changes of the raw data were determined for individual boxes (*B*). Scale bar, 1 cm. Individual images consisted of 128 frames and required a total of 5 s to collect (4 s) and store (1 s). After acquiring 3–6 control images, bipolar constant current cortical stimulation was used to evoke epileptiform afterdischarge activity (4 s, 60 Hz, biphasic—each phase 1.0 ms, peak-to-peak current amplitude 6 mA for stimulations 1–3 and 8 mA for stimulation 4). *b*, Normalized control image representing baseline activity before stimulation 1. The image serves as the control for *c* and *d*. The magnitude of optical changes ($\Delta R/R$, see methods) for all images in Fig. 1 are represented by the pseudocolour scale to the right of this image. Changes below zero are represented in black. *c*, Image of optical changes immediately after stimulation 2 (4 s, 6 mA). This stimulation was followed by the shortest of the four afterdischarge episodes (<4 s; see bottom left trace of *B*). The left-hand filled circle on the time axis in *B* marks the point when the image was obtained. The optical changes are greatest just above the recording electrode. The surrounding black cortical areas again represent regions where the optical changes are below baseline. *d*, Image obtained immediately after stimulation 4 (4 s, 8 mA). This elicited the longest of the four afterdischarge episodes (16 s; see bottom right trace of *B*). The right-hand filled circle on the time axis in *B* shows when this image was obtained. The evoked epileptiform activity after this stimulation was the only one of the four which triggered tongue and facial twitches. *B*, Normalized optical changes from the three boxes labelled in *Aa*. The arrows represent stimulus application for four seconds and the number beside the arrow is the constant current stimulation intensity in mA. At the bottom, two 20-s surface EEG traces obtained during stimulation 2 and 4 are shown. Optical changes between the stimulating electrodes (site 1) and next to the recording electrode (site 2) show a graded change to the different intensity and duration of the epileptiform afterdischarge activity. Other surrounding areas (such as site 3), have optical changes in the opposite direction (below baseline) compared with sites 1 and 2. These changes below the baseline are delayed when the afterdischarge activity lasts longer (compare site 3 after stimulations 2 and 4).

METHODS. A fibre-optic light regulated by a d.c. power supply (Lambda Inc.) was passed through a 695 longpass filter and a beam splitter to illuminate the cortical surface evenly. Images were acquired with a charge-coupled device camera (COHU 6500) fitted to the operating microscope. The cortex was stabilized with a glass footplate and the images were acquired at 30 Hz, and digitized at 8 bits (512 × 480 pixels) using Imaging Tech. Inc. Series 151 system. Geometrical transformations were applied to images to compensate for small amounts of patient motions²⁸. $\Delta R/R$ denotes a dimensionless quantity of magnitude of optical change. It is calculated by dividing the difference image (first control image —



subsequent image) by the first control image. All images were normalized to a control. For example, the normalized raw data (no digital enhancement) was used for determining the average optical change in specified regions or boxes (average size of boxes, 20 × 20 pixels or 1.5–2.5 mm²). For pseudocolour images, a linear low-pass filter removed high frequency noise and linear histogram transformations were applied²⁹. Noise was defined as the standard deviation of fluctuations in sequentially acquired normalized control images (0.003–0.009).

Our findings concur with studies showing large electrical surface potentials in the sensory cortex during finger movement²¹. There is a 10–30% increase in magnitude of the optical changes in the sensory cortex during tongue movement, which may reflect the results of sensory and motor cortex studies where cerebral blood flow increased by 10–30% during motor tasks²². These studies reflect a more general trend showing increases in neuronal activity in the mammalian brain tightly correlated with increases in blood flow, glucose metabolism, blood volume, and blood-brain barrier permeability²³.

Optical images were obtained from Broca's area and sensory cortex (Fig. 2*Ba*) while the patient silently viewed blank slides and while naming objects on slides presented every two seconds. Images obtained during this naming exercise show activation of the premotor cortex (baseline, Fig. 2*Bb*; naming, Fig. 2*Bc*) while the sites of speech arrest (sites 3 and 4) and palate tingling (site 1), identified by surface stimulation, yield optical signals

in the opposite direction (black areas, Fig. 2*Bc*). The area that is active during tongue movement is clearly different from the active area in the naming exercise (compare 2*Ba* and *Bc*). The optical changes in the premotor cortex during the naming exercise are similar to the cortical areas identified by positron emission tomography single-word processing studies^{24,25}. The optical changes were greatest in the anatomical area of cortex traditionally defined as Broca's area (the posterior portion of the inferior frontal gyrus²⁶, that is, sites 5, 6 and 7 in Fig. 2*Ba*) and not as expected in areas where electrical stimulation caused speech arrest (sites 3 and 4). Further definition of Broca's area may arise from optical imaging studies aimed at dissecting the fine structure of such cortical regions.

In three other patients, posterior essential language sites (Wernicke's areas) were identified with cortical stimulation mapping²⁰. Imaging during the naming exercises showed optical changes in these regions of the temporal lobe. In one patient,

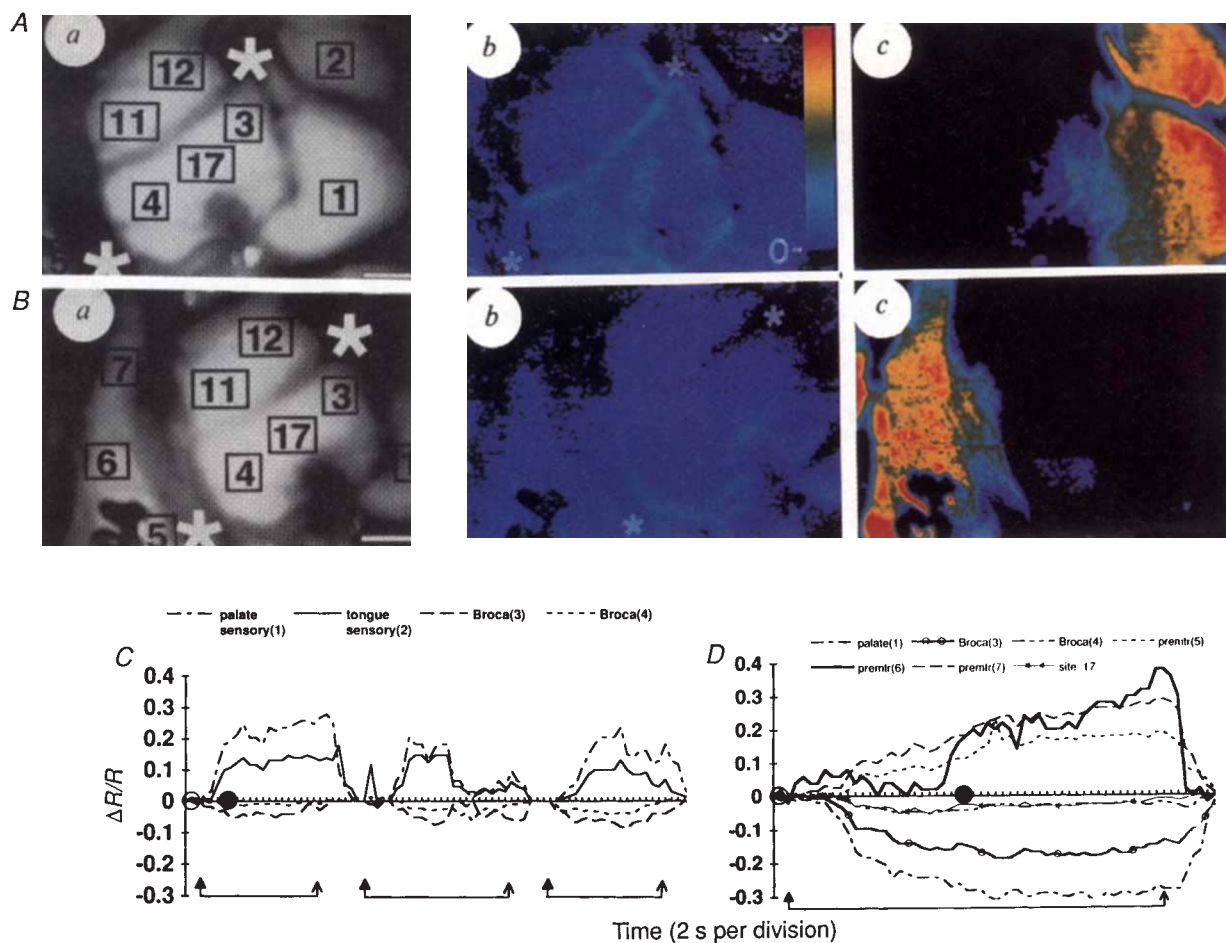
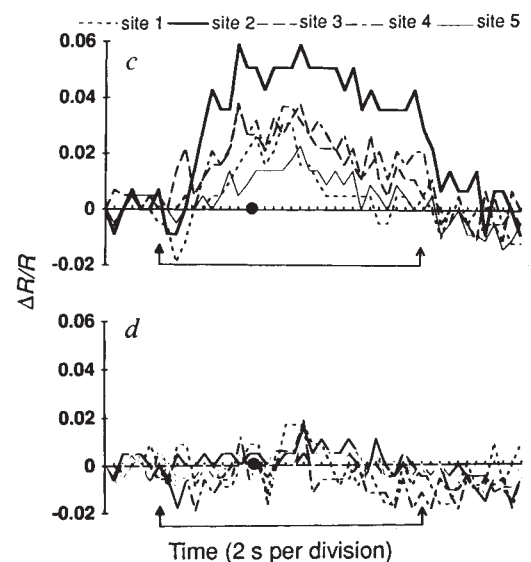
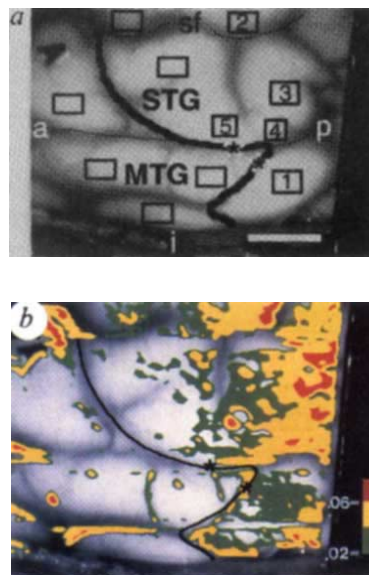


FIG. 3 *a*, Image of the cortical surface; anterior (a), inferior (i), posterior (p), Sylvian fissure (sf), superior temporal gyrus (STG) and middle temporal gyrus (MTG). Scale bar, 1 cm. Sites 1 and 2 were identified as essential for speech. At site 3, one naming error in three stimulation trials was made. Stimulation (7 mA) in all other unlabelled boxes did not cause errors in naming slides. The temporal lobe cortical tissue to the left of the thick line was surgically removed following cortical mapping of language and optical imaging, while testing the patient's language. As the surgical removal reached the asterisks (*—*, sites 4 and 5) language deteriorated. Images were collected as described in Fig. 2. *b*, Graphic overlay of optical changes in Wernicke's area during the naming task. The filled circles on the time axes in *c* and *d* mark when the image was obtained. Only optical changes with a $\Delta R/R > 0.02$ are represented on the pseudocolour scale. The largest optical changes are in the region of essential and secondary language sites and not in the more anterior cortex. In these non-essential regions, the optical changes are restricted to blood vessels, with the largest signals coming from the largest blood vessels (that is, the Sylvian fissure vessels in the top left of the figure). *c*, Normalized optical changes from the five language sites previously described (1–5). At these sites, optical



changes only occur during the naming task and not before. *d*, Normalized optical changes from the six sites not essential for language (unlabelled boxes, *a*). There are only fluctuations about the baseline at these more anterior sites in contrast to the language sites during the naming task. METHODS. See Fig. 1 legend.

FIG. 2 Anterior, left; posterior, right; superior, top and Sylvian fissure, bottom. Asterisks on the cortical surface serve as reference points between *Aa* and *Ba* and *Ba* and *b*. Scale bar, 1 cm. *Aa*, Numbered boxes represent sites where cortical stimulation (4 mA) evoked palate tingling (1), tongue tingling (2), speech arrest-Broca's areas (3, 4) and no response (11, 12, 17, 5). In three trials, an image was collected as an average of values (1 s, 32 frames) and stored (1 s) every 2 s at rest, during 40 s of tongue movement and during recovery. *Ab*, Control image before tongue movement. Open circle on time axis in *C* (near origin) marks when control images were collected. Image shows noise about the baseline at rest. The magnitude of optical change for the images is represented by the pseudocolour scale. *Ac*, Image collected during tongue movement. The filled circle on the time axis in *c* marks the time when the tongue movement image was obtained. *Ba*, Cortical surface from same subject with the image moved slightly forwards to incorporate more of the premotor cortex. In other premotor sites (6 and 7), no change in language was found during cortical stimulation (6 mA). The patient viewed blank slides (control) and was then required to name pictures appearing every 2 s. *Bb* and *c*, Images acquired during naming tasks. Control image (*Bb*) compared with naming image (*Bc*) shows that the maximum optical changes are located in the premotor region. Circles on the time axis in *D* mark when control images (open circle) and naming image (filled circle) were obtained. *C*, Normalized optical changes during tongue movement from each of the four areas with positive responses to cortical stimulation mapping (palate tingling, site 1; tongue tingling, site 2; speech arrest, sites 3 and 4). The closed arrowheads show when tongue movement began and open arrowheads when the movement ceased. The changes in the four areas were reproduced in all three trials and the largest positive changes are from the sensory cortex (sites 1 and 2). *D*, Normalized optical changes collected during the naming exercise from sites labelled in *Ba*. The cortical areas of maximal optical change are located in the premotor cortex (sites 5–7). Optical changes in the opposite direction occur in the sensory cortex (site 1) and at the sites of speech arrest (3 and 4). The premotor region also shows temporal differences in activation, with the centre area (site 6) being active earliest and the surrounding regions being active after a delay (sites 5 and 7). The closed arrowhead shows when the naming task began and open arrowhead when the task ceased.

METHODS. See Fig. 1 legend.

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Bicalyx is a natural homeotic floral variant

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INDUCED homeotic floral mutants in *Arabidopsis thaliana* and *Antirrhinum majus*^{1–3} are used to investigate the molecular mechanisms that establish floral organ identity. Here we describe *bicalyx*, a naturally occurring homeotic floral variant in *Clarkia concinna* (Onagraceae) that replaces petals with sepal-like structures. Typical *C. concinna* flowers have four sepals, four tri-lobed, bright pink petals, four stamens and a four-part ovary. *Bicalyx* flowers appear to have eight sepals, no petals and wild-type stamens and ovary with no reduction in fertility. All *bicalyx* organs on a plant are alike and have no developmental abnormalities, in contrast to many of the homeotic phenotypes described^{4–7}. *Bicalyx* demonstrates that a large morphological difference governed by a simple genetic change can become established in a natural plant population and suggests that even homeotic genes play a role in the evolution of morphological diversity in plants.

Bicalyx plants were found in a single locality at Point Reyes, California, north of San Francisco, and comprise 20–30% of the population of several hundred individuals. They were first observed in 1988 and have been present in a similar proportion each year since then. Both *bicalyx* and wild-type plants of the source population are self-fertile and highly self-pollinating. Nevertheless, seeds from one field specimen (of 45 studied) gave rise to both *bicalyx* and wild-type plants, indicating that insect pollinators visit *bicalyx* flowers.

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two essential language sites were identified by cortical stimulation at 7 mA (sites 1 and 2, Fig. 3a). A possible secondary language site (site 3) was also identified by the observation of a single naming error during three stimulations of the site. As the surgical removal of cortical tissue anterior to the thick line (Fig. 3a) neared sites 4 and 5 (*—* in Fig. 3a), the patient's language deteriorated. In Fig. 3b, optical changes during naming (as in Fig. 2 naming study) were superimposed on the image of the cortical surface. Before the surgical removal, optical changes were found at the essential (sites 1 and 2) and secondary (sites 3–5) language regions. The anterior cortical regions, without language disruption during cortical surface stimulation (unlabelled boxes Fig. 3a) or actual surgical removal, showed no significant optical changes during the naming exercise except along blood vessels. The essential and secondary language sites (sites 1–5; Fig. 3a), gave significant optical changes (Fig. 3c), whereas at the non-essential sites there were no significant optical changes in either direction (Fig. 3d). Cortical stimulation mapping only identifies essential cortex that must be preserved to limit language deficits after surgery. Our findings suggest that optical imaging may be able to identify both essential and secondary language areas that must be preserved during neurosurgical procedures to prevent language deficits.

Although the optical signals were relatively large, we encountered technical problems in obtaining these data including limited study time in the operating room (30–40 min), fluctuations in ambient light, and patient movement. Nevertheless, high resolution optical imaging of human epileptic and functional regions has the potential to allow studies of the fine structure of epileptic foci and cognitively evoked optical changes during motor, language, and memory tasks²⁷. □



FIG. 1 *Clarkia concinna* (Point Reyes) wild-type flower with tri-lobed petals, front and side views (left), and *bicalyx* flower, front and side views (right), at the same magnification.

Bicalyx organs resemble true sepals in size, shape and colour, having pointed apex and no lateral lobes. Like sepals, they are strongly reflexed after anthesis, are brownish in the distal regions owing to the presence of green chloroplasts and have numerous stomata. In venation pattern, number of cell layers, epidermal cell shape, and trichome features, they are intermediate between sepals and petals. The *bicalyx* phenotype clearly results from the imposition of a developmental program on the second whorl, which is usually expressed only in the first, and thus is properly considered homeotic.

We crossed *bicalyx* plants with wild-type plants from the source population and the three subspecies of *C. concinna*. All F_1 plants were like the wild-type parent and the F_2 segregated with a ratio of three tri-lobed to one *bicalyx* plant (Table 1),

indicating that the mutant phenotype is governed by a single recessive gene. *Bicalyx* was transferred by hybridization to the related *C. breweri* and also confers the characteristic phenotype on this background. Because no abnormalities or new phenotypes appeared in the diverse F_2 progenies, it is unlikely that *bicalyx* expression requires stabilization by modifiers, although the data do not rule out tightly linked genes.

Crinkled petal, another recessive variant producing second whorl organs that resemble sepals, was described in two related species from a different taxonomic section of *Clarkia* but was not originally recognized as homeotic^{8,9}. Wild-type petals of these species have a limb-and-claw structure quite unlike the tri-lobed *C. concinna* petals. The resemblance of the two mutant phenotypes presumably reflects the similarity of their sepals.

Bicalyx and *crinkled petal* join a small list of homeotic genes

TABLE 1 <i>Bicalyx</i> crosses					
Name	Parents	Wild type	<i>Bicalyx</i>	Model	χ^2
F_2	8852- <i>bcx</i> $\times$ 8852-wt	200	52	3:1	2.56
F_2	8852-wt $\times$ 8852- <i>bcx</i>	176	61	3:1	0.07
F_2	8852- <i>bcx</i> $\times$ 8740-wt	159	38	3:1	3.43
F_2	8740-wt $\times$ 8852- <i>bcx</i>	138	49	3:1	0.14
BC	(8852- <i>bcx</i> $\times$ 8740-wt) $\times$ 8852- <i>bcx</i>	22	26	1:1	0.33
F_2	8852- <i>bcx</i> $\times$ 8817-wt	148	47	3:1	0.08
F_2	8817-wt $\times$ 8852- <i>bcx</i>	129	47	3:1	0.27
F_2	8852- <i>bcx</i> $\times$ 8701-wt	79	29	3:1	0.20

Progeny segregations from crosses between *bicalyx* (*bcx*) from Point Reyes and wild-type (wt) plants from the same population (8852), and from subspecies *concinna* (8740), subspecies *raichei* (8817), and subspecies *automixa* (8701).

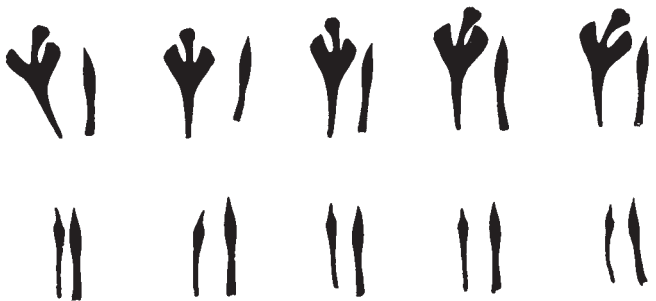


FIG. 2 Silhouettes of wild-type petals and sepals (top) and *bicalyx* organs and sepals (bottom), five flowers each, Point Reyes population.

(blind and green petal in *Petunia*, heptandra in *Digitalis*)¹⁰ affecting only second whorl floral organs. Most known homeotic genes modify at least two adjacent floral whorls. It has been proposed that their products act in concentric, partially overlapping zones at the floral apex, the combination of gene products acting in each zone determining the identity of organs arising there^{1,3}. If this is true, it seems that their effects can be in part reversed by the action of genes affecting only one whorl.

Homeotic mutants have been found and propagated in gardens^{11,12} but have almost never been reported as natural populations. The establishment of two homeotic mutants in annual *Clarkia* populations was probably possible because both are highly self-pollinating and neither reduces fertility. A peloric *Linaria* in which the normal upper petals were replaced by spurred lower petals was described by Linnaeus and may have

been abundant in its habitat, but since peloric *Linaria* was mostly seed-sterile it was probably a vegetatively perpetuated clone¹³.

The absence of deleterious pleiotropy or fitness-reducing epistatic interactions in *bicalyx* suggests that mutations with extensive morphological consequences can be successfully accommodated by plant developmental systems¹⁴. If such mutants were to become associated with chromosomal rearrangements reducing the fertility of hybrids between them and their progenitors, a process that has occurred repeatedly in *Clarkia*¹⁵, the new population would probably be accorded species status. Although the frequency of establishment of such mutations is unknown, *bicalyx* demonstrates that the kind of regulatory mutations being studied by plant developmental biologists may contribute to morphological diversification in nature. □

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Identification by mutagenesis of a Mg^{2+} -block site of the NMDA receptor channel

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THE *N*-methyl-D-aspartate (NMDA) receptor channel is highly permeable to Ca^{2+} but is blocked by Mg^{2+} in a voltage-dependent manner^{1–4}. These characteristics are essential for the NMDA receptor channel to mediate the induction of long-term potentiation of synaptic efficacy, a form of activity-dependent synaptic plasticity thought to underlie memory, learning and development^{5–8}. Recent studies have revealed the molecular and functional diversity of the NMDA receptor channel subunits, which are classified into the ϵ and ζ families according to the amino-acid sequence homology^{9–12}. Here we report that replacement by glutamine of asparagine 598 in putative transmembrane segment M2 of the ζ 1 subunit, strongly reduces the sensitivity of the heteromeric ϵ 2/ ζ 1 NMDA receptor channel to Mg^{2+} block. The corresponding mutation of the ϵ 2 subunit has a similar effect. Furthermore, the heteromeric ϵ 2/ ζ 1 NMDA receptor channel with the mutation on both subunits shows greatly reduced sensitivity to MK-801, a channel blocker of the NMDA receptor channel^{13,14}, but is still susceptible to inhibition by Zn^{2+} ^{15,16}. These findings suggest that the conserved asparagine residue in segment M2 constitutes a Mg^{2+} -block site of the NMDA receptor channel, and that the MK-801 site overlaps the Mg^{2+} site.

Site-directed mutagenesis has shown that arginine at position 586 in the putative transmembrane segment M2 of the α 2(GluR2) subunit determines the Ca^{2+} permeability of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-selective glutamate receptor (GluR) channel^{17–19}. The corresponding positions of the NMDA receptor channel subunits are occupied by asparagine^{9–12}. To test the idea that this

asparagine residue causes the NMDA receptor channel to be sensitive to Mg^{2+} block^{1,2}, we introduced a substitution mutation into the ϵ 2 and ζ 1 subunits of the mouse NMDA receptor channel^{10,12} (mutations ϵ 2-N589Q and ζ 1-N598Q; Fig. 1). A cluster of negatively charged residues at the amino-terminal side of segment M2 of the ζ 1 subunit¹⁰ was also replaced by the corresponding amino-acid residues of the α 1 subunit of the AMPA-selective glutamate receptor channel²⁰ (mutation ζ 1-ZAZ; Fig. 1).

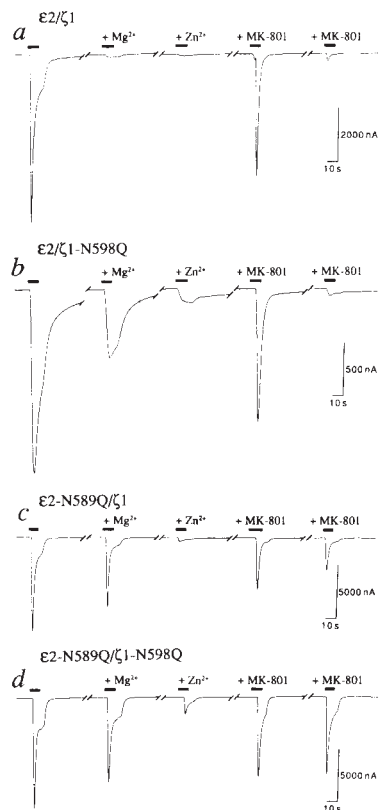
Effects of these mutations on channel properties were examined after expression in *Xenopus* oocytes, by co-injection of the wild-type or mutant ϵ 2 subunit-specific mRNA and the wild-type or mutant ζ 1 subunit-specific mRNA. Figure 2 shows the current responses of the resulting heteromeric NMDA receptor channels to 10 μ M L-glutamate plus 10 μ M glycine, at -70 mV membrane potential, in normal frog Ringer's solution. The wild-type ϵ 2/ ζ 1 NMDA receptor channel is effectively blocked by 1 mM Mg^{2+} , 100 μ M Zn^{2+} and 1 μ M (+)-MK-801. In contrast, the mutant ϵ 2/ ζ 1-N598Q channel gave a large

	M2
ϵ 2	DGREPGGPSFTIGKAIWLLGLVFNNSVPVQ
ϵ 2-N589Q	-----Q-----
ζ 1	NSEEEEEEDALTSSAMWFSWGLVLLNSGIGEG
ζ 1-N598Q	-----Q-----
ζ 1-ZAZ	--TSDQSN-----N-----

FIG. 1 Substitution mutations introduced into the ϵ 2 and ζ 1 subunits of the mouse NMDA receptor channel^{10,12}. Amino-acid sequences of the mutagenized region encompassing putative transmembrane segment M2 are shown. Bars represent residues identical to those above in the aligned position.

METHODS. Site-directed mutagenesis was carried out by a two-step polymerase chain reaction (PCR)²⁵ using appropriate synthetic oligonucleotides and DNA fragments derived from the plasmids pBKSA ϵ 2 (ref. 12) and pBKSA ζ 1 (ref. 10). The mutagenized 353-bp *Cfr*10I-*Sph*I and 322-bp *Fsp*I-*Bln*I fragments obtained from PCR amplified products were substituted for the corresponding segments of pBKSA ϵ 2 and pBKSA ζ 1, respectively. The nucleotide sequences of the constructed plasmids differ from the respective parental plasmids as follows (the residue numbers^{10,12} and the substituted nucleotides): pBKSA ϵ 2-N589Q, 1765C, 1767G; pBKSA ζ 1-N598Q, 1792C, 1794G; pBKSA ζ 1-ZAZ, residues 1726–1743, ACCAGTGACCAAGTCAAT.

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current response in the presence of 1 mM Mg^{2+} . But the response was effectively suppressed by 100 μM Zn^{2+} and 1 μM (+)-MK-801. The mutation $\epsilon 2$ -N589Q similarly reduced the sensitivity of the heteromeric channel to Mg^{2+} block without affecting the sensitivity to Zn^{2+} . In contrast to the $\epsilon 2/\zeta 1$ and $\epsilon 2/\zeta 1$ -N598Q

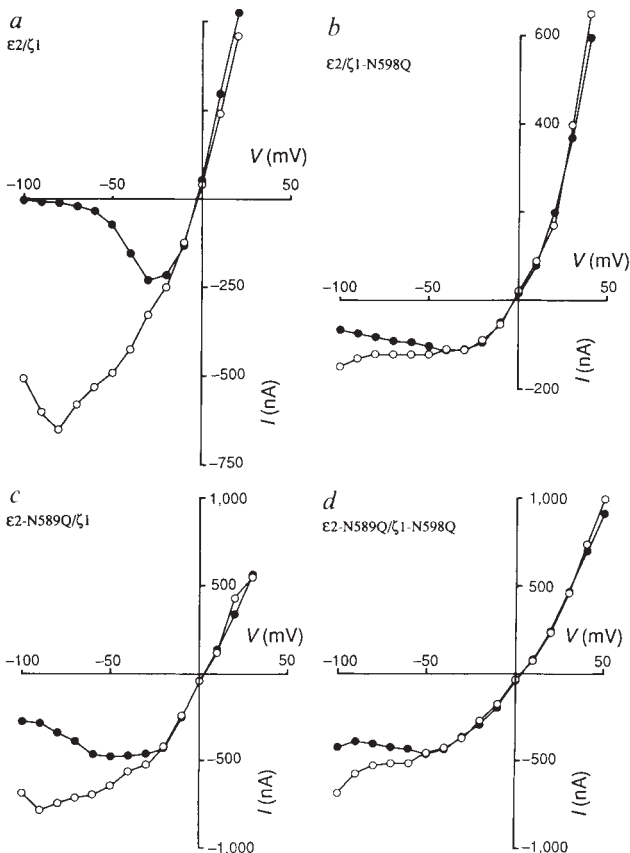


FIG. 2 Sensitivities to non-competitive antagonists of mutant NMDA receptor channels. The heteromeric $\epsilon 2/\zeta 1$ (a), $\epsilon 2/\zeta 1$ -N598Q (b), $\epsilon 2$ -N589Q/ $\zeta 1$ (c) and $\epsilon 2$ -N589Q/ $\zeta 1$ -N598Q (d) NMDA receptor channels were expressed in *Xenopus* oocytes by injection of respective mRNAs. Current responses to 10 μM L-glutamate plus 10 μM glycine were measured in normal frog Ringer's solution at -70 mV membrane potential. Effects of non-competitive antagonists were examined in Ringer's solution containing 1 mM $MgCl_2$, 100 μM $ZnCl_2$ or 1 μM (+)-MK-801. The sensitivity to MK-801 was evaluated after successive application because the drug acts as an open-channel blocker^{13,14}. Inward current is represented downwards. The duration of bath application of agonists is indicated by bars, without taking into account the dead-space time in the perfusion system (~ 2 s).

METHODS. Subunit-specific mRNAs were synthesized *in vitro* as described^{10,12}. *Xenopus* oocytes were injected with wild-type or mutant $\epsilon 2$ subunit-specific mRNA (~ 19 ng per oocyte) and the wild-type or mutant $\zeta 1$ subunit-specific mRNA (~ 13 ng per oocyte). Whole-cell currents were recorded after 2–3 days incubation with a conventional two-micropipette voltage clamp as described²⁰.

channels, the $\epsilon 2$ -N589Q/ $\zeta 1$ channel showed large current responses after repetitive application of (+)-MK-801. The heteromeric $\epsilon 2$ -N589Q/ $\zeta 1$ -N598Q channel was highly resistant to block by Mg^{2+} and (+)-MK-801, but was still sensitive to Zn^{2+} .

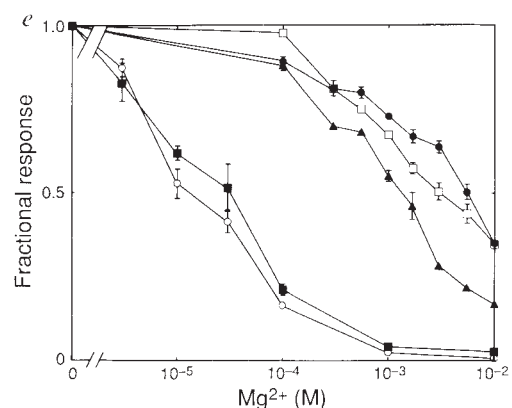
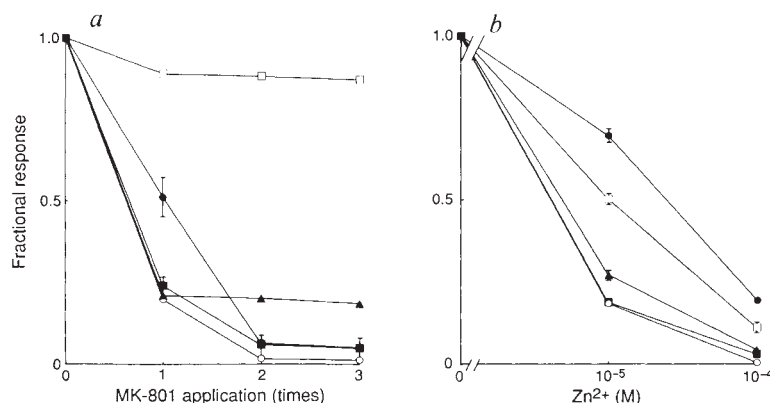


FIG. 3 Effects of substitution mutations on the sensitivity to Mg^{2+} block. Current responses to 10 μM L-glutamate plus 10 μM glycine were measured in Ba^{2+} -Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$ and 10 mM HEPES-NaOH (pH 7.2). a–d, Current (I)-voltage (V) relationships in the presence (filled circles) and absence (open circles) of 1 mM Mg^{2+} . Responses were recorded from an oocyte implanted with the heteromeric $\epsilon 2/\zeta 1$ (a), $\epsilon 2/\zeta 1$ -N598Q (b), $\epsilon 2$ -N589Q/ $\zeta 1$ (c) or $\epsilon 2$ -N589Q/ $\zeta 1$ -N598Q (d) NMDA receptor channel. e, Effects of Mg^{2+} concentrations on the responses of the $\epsilon 2/\zeta 1$ (○), $\epsilon 2/\zeta 1$ -N598Q (●), $\epsilon 2$ -N589Q/ $\zeta 1$ (▲), $\epsilon 2$ -N589Q/ $\zeta 1$ -N598Q (□) and $\epsilon 2/\zeta 1$ -ZAZ (■) channels. Each point represents the mean $\pm$ s.e.m. of measurements on 3 oocytes at -70 mV membrane potential. Current responses obtained in the absence of Mg^{2+} were 40–1250 nA (○), 158–478 nA (●), 394–1156 nA (▲), 120–606 nA (□) and 20–505 nA (■).

FIG. 4 Effects of substitution mutations on the sensitivities to MK-801 and Zn^{2+} . Current responses to 10 μ M L-glutamate plus 10 μ M glycine of the $\epsilon 2/\zeta 1$ ($\circ$), $\epsilon 2/\zeta 1$ -N598Q ($\bullet$), $\epsilon 2$ -N589Q/ $\zeta 1$ ($\blacktriangle$), $\epsilon 2$ -N589Q/ $\zeta 1$ -N598Q ($\square$) and $\epsilon 2/\zeta 1$ -ZAZ ($\blacksquare$) channels were measured in Ba^{2+} -Ringer's solution at -70 mV membrane potential. Each point represents the mean $\pm$ s.e.m. of measurements on 3 oocytes. **a**, Sensitivity to sequential treatment of 1 μ M (+)-MK-801. Current responses obtained before MK-801 treatment were 112–316 nA ($\circ$), 172–370 nA ($\bullet$), 152–321 nA ($\blacktriangle$), 166–706 nA ($\square$) and 59–94 nA ($\blacksquare$). **b**, Sensitivity to various concentrations of Zn^{2+} . Current responses obtained in the absence of Zn^{2+} were 251–538 nA ($\circ$), 84–336 nA ($\bullet$), 438–628 nA ($\blacktriangle$), 144–350 nA ($\square$) and 113–713 nA ($\blacksquare$).



The effects of these mutations on the sensitivity to Mg^{2+} block were examined quantitatively in Ba^{2+} -Ringer's solution, to minimize the contamination of secondarily activated Ca^{2+} -dependent Cl^{-} currents²¹. Figure 3a–d shows current-voltage relationships of the wild-type and mutant heteromeric channels in the presence (filled circles) and absence (open circles) of 1 mM Mg^{2+} . Mg^{2+} inhibited the current responses of the wild-type $\epsilon 2/\zeta 1$ channel in a voltage-dependent manner (Fig. 3a), as observed for native NMDA receptor channels^{1,2}. The point mutation of the asparagine residue in either or both of the subunits, strongly reduced the sensitivity of the heteromeric channels to Mg^{2+} , and the mutant channels were active even at -100 mV membrane potential (Fig. 3b–d). The mutation seems to also affect the current-voltage relations in the absence of Mg^{2+} . The extents of inhibition by various concentrations of Mg^{2+} were compared at -70 mV membrane potential (Fig. 3e). The channel activities of the wild-type $\epsilon 2/\zeta 1$ channel and the $\epsilon 2/\zeta 1$ -ZAZ channel were reduced to 50% by ~ 20 μ M Mg^{2+} and were almost completely suppressed by the physiological concentration of Mg^{2+} (~ 1 mM). But Mg^{2+} concentrations about two orders of magnitude higher were required to suppress the mutant $\epsilon 2/\zeta 1$ -N598Q, $\epsilon 2$ -N589Q/ $\zeta 1$ and $\epsilon 2$ -N589Q/ $\zeta 1$ -N598Q channels.

Repetitive application of 1 μ M (+)-MK-801 (an open channel blocker of the NMDA receptor channel^{13,14}) virtually suppressed the wild-type $\epsilon 2/\zeta 1$ channel (Fig. 4a). Similarly, the channel activities of the mutant $\epsilon 2/\zeta 1$ -N598Q and $\epsilon 2$ -N589Q/ $\zeta 1$ channels were strongly reduced by the (+)-MK-801 treatment; however, the mutant $\epsilon 2$ -N589Q/ $\zeta 1$ -N598Q channel was highly active after three successive applications of (+)-MK-801. We also examined the effect of Zn^{2+} , reported to induce a non-competitive inhibition of the NMDA receptor channel independently of voltage^{15,16}. The sensitivities of the heteromeric channels to 10 μ M Zn^{2+} were reduced by these point mutations, but the mutant channels were strongly inhibited by 100 μ M Zn^{2+} (Fig. 4b).

The NMDA receptor channel is essential for the induction of long-term potentiation^{5,7}. Mg^{2+} block is the key to the depolarization-dependent activation of the NMDA receptor

channel (the basis of an activity-dependent change of synaptic efficacy, thought to underlie learning, memory and development^{1–8}). Here we have shown that replacement by glutamine of the conserved asparagine in segment M2 of either of the $\epsilon 2$ and $\zeta 1$ subunits abolishes the block of the heteromeric NMDA receptor channel by the physiological concentration of Mg^{2+} . Asparagine and glutamine are similar in chemical nature and differ in size only by one hydrocarbon group. Drastic change in the sensitivity to Mg^{2+} caused by only a subtle change in structure suggests that the asparagine residue is a major constituent of the Mg^{2+} block site. The same substitution introduced into both subunits strongly reduces the sensitivity of the heteromeric channel to MK-801, suggesting that the site of MK-801 block overlaps, or is the same as, the Mg^{2+} site. There is strong evidence that Mg^{2+} produces a voltage-dependent block of the channel by binding a site deep within the ionophore²², thus our results are consistent with the view that segment M2 constitutes the transmembrane ion channel of the NMDA receptor channel. When the amino-acid sequences of homologous glutamate receptor channel subunits are compared^{10–12}, the asparagine conserved among NMDA receptor channel subunits corresponds in position to glutamine and arginine of the AMPA-selective GluR channel subunits that determine the divalent cation permeability of the channel^{17–19}. This suggests the common channel structure for the NMDA and non-NMDA receptor channels.

We have previously shown that the $\epsilon 3/\zeta 1$ NMDA receptor channel is less sensitive to Mg^{2+} than the $\epsilon 1/\zeta 1$ and $\epsilon 2/\zeta 1$ channels¹². As the asparagine is conserved in these subunits, there are probably additional residues involved in interaction with Mg^{2+} . The observation that sensitivity to Zn^{2+} inhibition is only slightly affected by the substitution mutation of the Mg^{2+} site is consistent with the proposal that Mg^{2+} and Zn^{2+} act on different sites¹⁶. Although the main effect of Zn^{2+} on the NMDA receptor channel is voltage-insensitive, a small portion of the Zn^{2+} block is voltage-dependent^{23,24}. The slight decrease in the sensitivity to Zn^{2+} of the mutant channels can be explained if the voltage-dependent block by Zn^{2+} is caused by binding to the Mg^{2+} site. □

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Nitric oxide release from a single cell measured *in situ* by a porphyrinic-based microsensor

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NITRIC oxide is an important bioregulatory molecule, being responsible, for example, for activity of endothelium-derived relaxing factor (EDRF)¹⁻⁴. Acute hypertension⁵, diabetes⁶, ischaemia⁷ and atherosclerosis⁸ are associated with abnormalities of EDRF. Nitric oxide is thought to be a retrograde messenger in the central nervous system⁹. The technology is not yet available for rapid detection of NO released by a single cell in the presence of oxygen and/or nitrite, so the release, distribution and reactivity of endogenous NO in biological systems cannot be analysed. Here we describe a porphyrinic microsensor that we have developed and applied to monitoring NO release in a microsystem. We selectively measured *in situ* the NO released from a single cell with a response time of less than 10 ms. The microsensor consists of p-type semi-conducting polymeric porphyrin and a cationic exchanger (Nafion) deposited on a thermally sharpened carbon fibre with a tip diameter of $\sim 0.5 \mu\text{m}$. The microsensor, which can be operated in either the amperometric or voltammetric mode, is characterized by a linear response up to $300 \mu\text{M}$ and a detection limit of 10 nM . Nitric oxide at the level of 10^{-20} mols can be detected in a single cell.

We deposited electrochemically a thin layer of polymeric porphyrin on carbon fibres. Carbon fibres are mechanically strong and can be controllably miniaturized for single-cell applications (Fig. 1). We have shown previously that highly conductive polymeric metalloporphyrins are catalysts for electrochemical oxidation of certain small molecules¹⁰ and now use the high current generated at 0.63 V during catalytic electrochemical oxidation of NO as an analytical signal for the microsensor. To discriminate against NO_2^- , the porphyrinic catalyst is covered with Nafion. The negatively charged Nafion is highly permeable to NO but prevents diffusion of anions like NO_2^- to the porphyrinic surface.

The polymeric film was obtained from monomeric tetrakis(3-methoxy-4-hydroxyphenyl)porphyrin, TMHPP, with nickel as the central metal, synthesized as described¹¹. We deposited poly-TMHPP-Ni from a solution of 0.1 M NaOH containing monomeric porphyrin, either by constant-potential electrolysis at 0.8 V or by continuous scan cyclic voltammetry (Fig. 2). The porphyrin surface coverage, Γ , was monitored by observing the

Ni(II)/Ni(III) redox couple at 0.52 V (optimal $\Gamma = 0.8\text{--}1.5 \text{ nmol cm}^{-2}$). Because the Ni(II)/Ni(III) reaction requires diffusion of OH^- to neutralize a charge generated in the poly-TMHPP-Ni and OH^- cannot diffuse through Nafion, the absence of the Ni(II)/Ni(III) voltammetric peaks in 0.1 M NaOH was used to demonstrate the integrity of the Nafion film coverage.

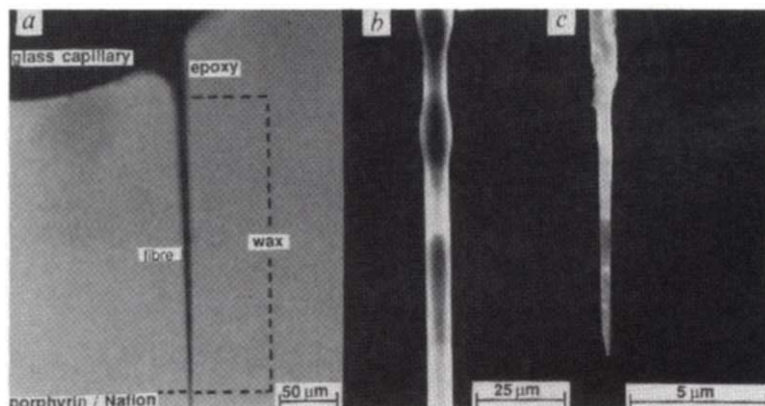
We used differential pulse voltammetry, DPV, or amperometry to monitor NO. DPV of NO on poly-TMHPP-Ni without Nafion shows a peak at 0.63 V in buffer pH 7.4 (Fig. 3a). DPV of a solution of $1 \mu\text{M}$ NO and $20 \mu\text{M}$ NO_2^- shows a single peak at 0.80 V (Fig. 3b). The peak current is three times higher than that observed at 0.63 V for NO alone. This indicates that the oxidation of NO_2^- and NO occur at a similar potential, but the current increase is not proportional to the concentration of NO_2^- . The NO peak current with the Nafion-coated sensor is observed at 0.64 V (Fig. 3c). Although the current is lower, Nafion coverage provides high selectivity against NO_2^- . We observed only a 1% increase in current and no potential change for oxidation of $1 \mu\text{M}$ NO in the presence of $20 \mu\text{M}$ NO_2^- (Fig. 3d). Electrochemical oxidation of NO on the sensor involves only one-electron transfer (1.02 ± 0.03 faradays measured by coulometry; $n = 7$) leading to the formation of NO^+ ($\text{NO} \cdot - e^- \rightarrow \text{NO}^+$), a known intermediate in the chemical oxidation of NO (ref. 12). NO_2^- is not produced on the surface of porphyrinic catalyst covered with Nafion. This indicates that the negatively charged Nafion stabilizes NO^+ . Oxidation of NO on porphyrinic film without Nafion involves 2.75 ± 0.22 faradays ($n = 7$), which indicates a more complicated pattern of reactions leading to the formation of NO_2^- and its further oxidation to NO_3^- .

We observed a linear relationship between current and NO concentration up to $300 \mu\text{M}$ ($r = 0.994$; slope, $2.05 \text{ nA per } \mu\text{M}$; $n = 21$). The response time (time for the signal increase from 10 to 75%) in the amperometric mode is less than 10 milliseconds. The detection limit calculated at a signal-to-noise ratio of 3 is 20 nM for DPV and 10 nM for the amperometric method. In a volume equivalent to that of an average single cell (10^{-12} litres), about 10^{-2} attomol (10^{-20} mol) of NO can be detected. The detection limit of the sensor is 2–4 orders of magnitude lower than the estimated amount of NO released per single cell ($1\text{--}200$ attomol per cell)^{13,14}.

Experiments in cell culture medium show a stable background and fast response to injected NO (Fig. 4a,b). The current observed indicates that the initial concentration around the sensor is 230 nM and decreases to 40 nM after 17 s mainly as a result of depletion of NO by diffusion and also reaction with O_2 .

Ring segments from porcine aorta (2–3 mm wide) and porcine aorta endothelial cell culture were prepared according to procedures described previously¹⁵. Using a computer-controlled

FIG. 1 a, Microscopic photograph of a carbon-fibre microsensor; b, electron scanning micrograph (ESM) of the part of the microsensor covered with a coat of isolating wax-rosin mixture; c, ESM of a thermally sharpened tip covered with poly-TMHPP-Ni. Microsensors were produced by threading carbon fibre ($7 \mu\text{m}$) through the pulled end of a capillary tube with $\sim 1 \text{ cm}$ left protruding. Nonconductive epoxy was put at the glass/fibre interface. When the epoxy drawn into the tip of the capillary had dried, the carbon fibre was sealed in place. The carbon fibre was sharpened by gradual burning (propane-air microburner $1,300\text{--}1,400^\circ\text{C}$) as described²⁰. The sharpened fibre was immersed in melted wax-rosin (5:1) at a controlled temperature for 5–15 s and, after cooling, was sharpened again. The flame temperature and the distance of the fibre from the flame needs to be carefully controlled. The resulting electrode is a slim cylinder with a small diameter rather than a short taper, a geometry that aids in implantation and increases the active surface area. ESM shows that the wax is burned roughly to the top of the sharpened tip. The tip (length $2\text{--}6 \mu\text{m}$) is the only part of the carbon fibre where electrochemical processes can occur. For the sensor to be implanted into a cell, this length



must be less than the cell thickness. The unsharpened end of the fibre was attached to a copper wire lead with silver epoxy.

micropositioner (0.2 mm *X-Y-Z* resolution), the microsensor can be implanted into a single cell or placed on the surface of the cell membrane, or kept at a controlled distance from the cell membrane. We placed one microsensor on the surface of the endothelium cell in the aortic ring, implanted another into the smooth muscle cell, then injected 2 nmol bradykinin into

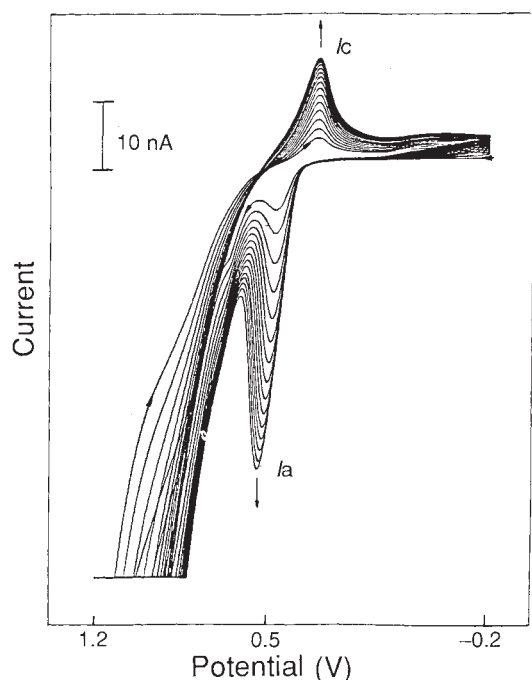


FIG. 2 The growth patterns for poly-TMHP-Ni, deposited from 5×10^{-4} M TMHP-Ni, 0.1 M NaOH solution by continuous scan cyclic voltammetry from -0.2 to 1.2 V on a carbon fibre microelectrode ($16 \mu\text{m}^2$ surface area). Peaks *a*, *c* correspond to the oxidation of Ni(II) to Ni(III) and reduction of Ni(III) to Ni(II), respectively, in the film. Surface coverage, Γ , is calculated from the charge transferred under process *a* ($\Gamma = 0.8 \text{ nmol cm}^{-2}$) and depends on the initial concentration of TMHP-Ni, electrolysis time and potential. Sensor fabrication is accomplished by dipping in the Nafion (solution 5% w/w in alcohol) for 15–20 s. The completed sensor is left to dry (5 min) and stored in buffer at pH 7.4.

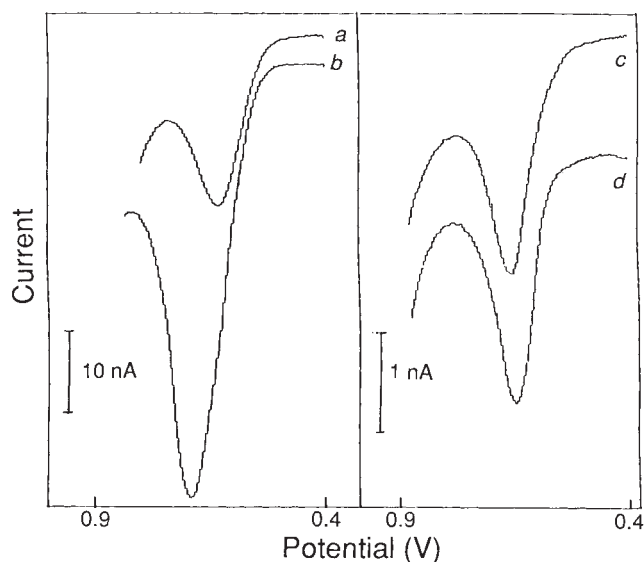


FIG. 3 Differential pulse voltammograms (sensor-working electrode, saturated calomel electrode-reference electrode and platinum wire-auxiliary electrode; pulse amplitude 40 mV; phosphate buffer, pH 7.4) obtained for oxidation of *a*, $1 \mu\text{M}$ NO on poly-TMHP-Ni; *b*, $1 \mu\text{M}$ NO and $20 \mu\text{M}$ NO_2^- on poly-TMHP-Ni; *c* and *d*, same conditions as for *a* and *b*, respectively, but on poly-TMHP-Ni covered with Nafion.

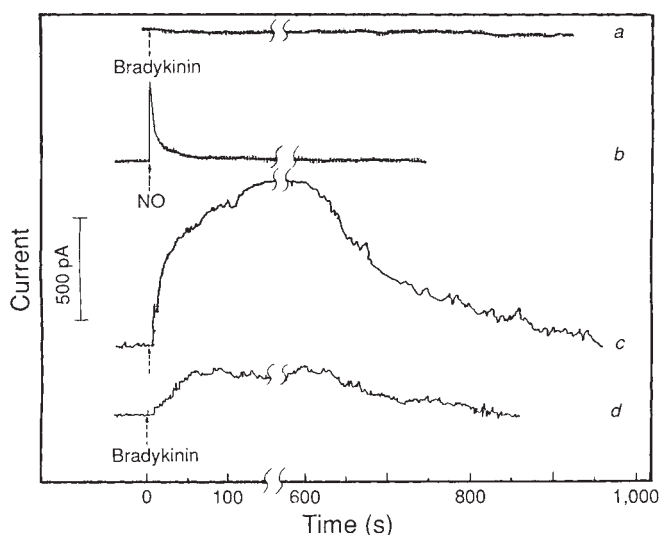


FIG. 4 Amperometric detection of NO by microsensor (alternating current was measured at constant potential of 0.75 V modulated with 40 mV pulse in time intervals of 0.5 s); *a*, background measured in cell culture medium at 37°C (Dulbecco's modified Eagle medium, 100 mg l^{-1} D-glucose, 2 mM glutamine, 110 mg l^{-1} sodium pyruvate, 15% controlled process serum replacement type I); no change of the background was observed after addition of 50 nmol bradykinin to 5 ml cell culture medium. *b*, NO (2 nmol) was injected by microsyringe into cell culture medium 5 mm from the microsensor; *c*, microsensor was placed on the surface of the single endothelial cell in porcine aorta then 2 nmol bradykinin was injected to the cell culture medium in the area of the cell; *d*, microsensor was implanted in a single smooth muscle cell in the aorta. Current was measured after injection of bradykinin simultaneously with *c*.

the medium near the endothelium cell. After 5 ± 0.5 s ($n = 7$), NO release was detected and a steady increase of surface concentration to a plateau at 450 ± 40 nM was observed after 150 s (Fig. 4c). After 16 min, the surface concentration of NO decreased to zero. No significant difference in NO surface concentration was found for an endothelial cell from a cell culture (430 ± 40 nM; $n = 7$). NO was detected in a single smooth muscle cell within 8.0 ± 0.5 s ($n = 7$) after injection of bradykinin and a maximum concentration (130 ± 10 nM, $n = 7$) was observed after 90 s (Fig. 4d).

The characteristics of this sensor, including size, detection limit, linear range, response time, selectivity and applicability to *in situ* measurements cannot be found in any other method for measuring NO (refs 16, 17). We found that the sensor is about three orders of magnitude more sensitive to NO than to catecholamines which can also be detected by electrochemical methods^{18,19}. Therefore the sensor can be applied without additional modification for NO detection in the brain if the concentration of catecholamines like dopamine is lower than 10^{-5} M. While this work describes the production and use of a microsensor for use in small environments, such as single cells, the same plating techniques can be applied to a larger support to produce a convenient macrosensor for tissue and cell culture studies. □

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Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca^{2+} flux but not mitogenesis

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STIMULATION of certain receptor tyrosine kinases results in the tyrosine phosphorylation and activation of phospholipase C_γ (PLC γ), an enzyme that catalyses the hydrolysis of phosphatidylinositol (PtdIns)^{1–8}. This hydrolysis generates diacylglycerol and free inositol phosphate, which in turn activate protein kinase C and increase intracellular Ca^{2+} , respectively. PLC γ physically associates with activated receptor tyrosine kinases, suggesting that it is a substrate for direct phosphorylation by these kinases^{7–10}. Here we report that a fibroblast growth factor (FGF) receptor with a single point mutation at residue 766 replacing tyrosine with phenylalanine fails to associate with PLC γ in response to FGF. This mutant receptor also failed to mediate PtdIns hydrolysis and Ca^{2+} mobilization after FGF stimulation. However, the mutant receptor phosphorylated itself and several other cellular proteins, and it mediated mitogenesis in response to FGF. These findings show that a point mutation in the FGF receptor selectively eliminates activation of PLC γ and that neither Ca^{2+} mobilization nor PtdIns hydrolysis are required for FGF-induced mitogenesis.

It has been shown that the FGF receptor can phosphorylate itself on Tyr 766 and that a recombinant SH2 domain (src-homologous domain 2) of PLC γ can bind to a tryptic fragment of the receptor containing this tyrosine¹¹. We reasoned that if Tyr 766 were the main site of interaction between the FGF receptor and PLC γ , a point mutation at this residue might produce a receptor with a selective impairment in the PtdIns turnover pathway. To determine the specificity of the interaction between the FGF receptor and PLC γ we synthesized peptides encompassing the two conserved tyrosines in the C-terminal tail of the receptor, Tyr 766 and Tyr 776, and asked whether they could block the association of PLC γ with recombinant FGF receptors *in vitro*^{12–14}. A peptide of nine amino acids that was phosphorylated on Tyr 766 (Y766P) blocked the association of

PLC γ with the FGF receptor at 10 μM , the lowest concentration tested (Fig. 1). The unphosphorylated version of this peptide (Y766U) partially blocked PLC γ association but only at much higher concentrations (200 μM). A peptide phosphorylated at Tyr 776 failed to block PLC γ association (data not shown). A scrambled phosphopeptide (Y766PS) also failed to block PLC γ association (Fig. 1). These results suggested that Tyr 766, when phosphorylated, was directly involved in the binding of PLC γ to the FGF receptor and that Tyr 766 and its flanking sequences constituted the major site of PLC γ association with the FGF receptor.

To test the functional significance of PLC γ binding to Tyr 766, we made a mutant FGF receptor in which Tyr 766 was replaced by phenylalanine (Y/F766). The Y/F766 mutant receptor and the wild-type receptor were then transfected into rat L6 myoblasts that do not express endogenous FGF receptors¹⁵. FGF treatment of cells expressing the wild-type FGF receptor enhanced receptor association of PLC γ , (Fig. 2a). But in two independent cell lines expressing the Y/F766 FGF receptor (Y/F766-1 and Y/F766-2), there was no detectable ligand-induced receptor association of PLC γ , despite the fact that more receptors were immunoprecipitated from these cells (Fig. 2a). More receptor is immunoprecipitated from the Y/F766 cell lines because these cells express around three times more receptor protein than the wild-type cell line (data not shown). We have recently isolated and evaluated an additional Y/F766 mutant cell line that expresses a similar amount of receptor protein as the wild-type clone. In this cell line the mutant FGF receptor behaved the same as in the clones that had more receptors.

Paralleling receptor association, tyrosine phosphorylation of PLC γ by the Y/F766 mutant receptor was dramatically decreased or absent compared with the wild-type receptor and there was no increase in PtdIns turnover in response to FGF stimulation (Fig. 2b and c). (A small amount of baseline tyrosine phosphorylation of PLC γ was seen in multiple experiments using several cell lines, including another vector-transfected cell line, and probably represents phosphorylation of PLC γ by kinases other than the FGF receptor.) These results further establish that Tyr 766 is necessary for ligand-induced association

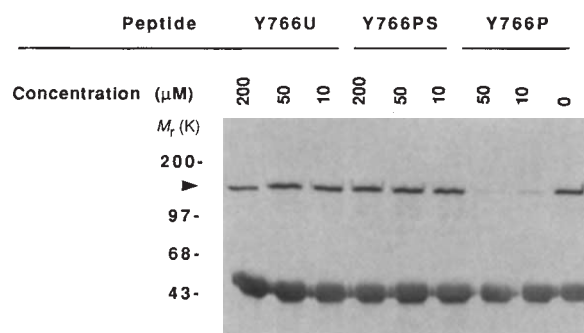


FIG. 1 A synthetic FGF receptor phosphopeptide encompassing Y766 blocks association of PLC γ with the FGF receptor tyrosine kinase *in vitro*. Peptides were synthesized based on the sequence of nine amino acids surrounding Y766 of the human FGF receptor¹⁴. Peptide Y766P (SNQEY₇₆₆LDLS) and a scrambled peptide, peptide Y766PS (Y₇₆₆DSLQSELN), were synthesized using phosphotyrosine; peptide Y766U (SNQEYLDLS) was synthesized using unphosphorylated tyrosine. These peptides were then tested for their ability to block the association of PLC γ to recombinant FGF receptors *in vitro*. Arrowhead indicates receptor-associated PLC γ .

METHODS. Peptides were synthesized and receptor association was assayed as described¹³. Recombinant chicken FGF receptor produced in a baculovirus expression system was immunoprecipitated from insect cell lysates using a polyclonal FGF receptor antibody (unpublished). The immunoprecipitated receptor was autophosphorylated *in vitro* and incubated with BALB/c 3T3 cell lysates that were preincubated with or without one of the synthetic peptides. The resultant receptor immunoprecipitates were analysed for the presence of co-immunoprecipitated PLC γ by immunoblot with anti-PLC γ antibodies (UBI).

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of PLC γ with the FGF receptor *in vivo* and suggest that receptor association is required for the phosphorylation and activation of PLC γ .

Many peptide growth factors, including FGF, stimulate a rapid increase in cytosolic Ca²⁺ concentration¹⁶. This process occurs in two phases, the first by transient release of Ca²⁺ from intracellular stores and the second by sustained entry of Ca²⁺ from the extracellular space. The release of Ca²⁺ from intracellular stores is probably mediated by inositol trisphosphate, and we have recently shown that Ca²⁺ entry from the extracellular space might also be mediated by a PtdIns metabolite¹⁷. In cells expressing the wild-type FGF receptor, FGF stimulated both phases of Ca²⁺ mobilization (Fig. 3a). But in cells expressing the Y/F766 mutant receptor, FGF failed to stimulate increased intracellular Ca²⁺ from either intracellular stores or the extracel-

lular space (Fig. 3b). Cells expressing either the wild-type or the mutant receptor had a normal Ca²⁺ response to platelet-derived growth factor (PDGF) (data not shown). These data strongly suggest that PtdIns turnover is required for FGF-induced Ca²⁺ mobilization from either intracellular stores or from the extracellular space.

Despite deficient interactions with PLC γ , the Y/F766 FGF receptor mutant was a functional receptor tyrosine kinase capable of responding to ligand stimulation. Both the wild-type and mutant FGF receptors were autophosphorylated in response to FGF (Fig. 4a) and both receptors mediated the phosphorylation of other cellular proteins after FGF stimulation (Fig. 4b, closed arrows). Two proteins (M_r 145,000 and 90,000) even seem

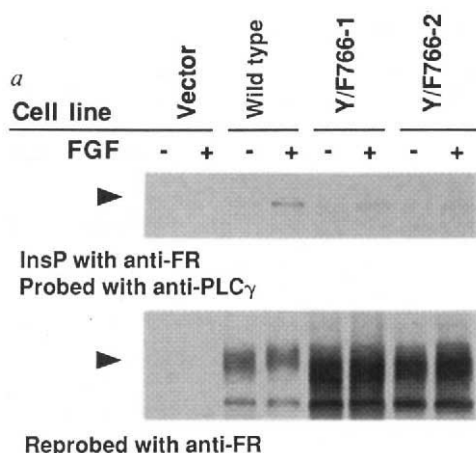
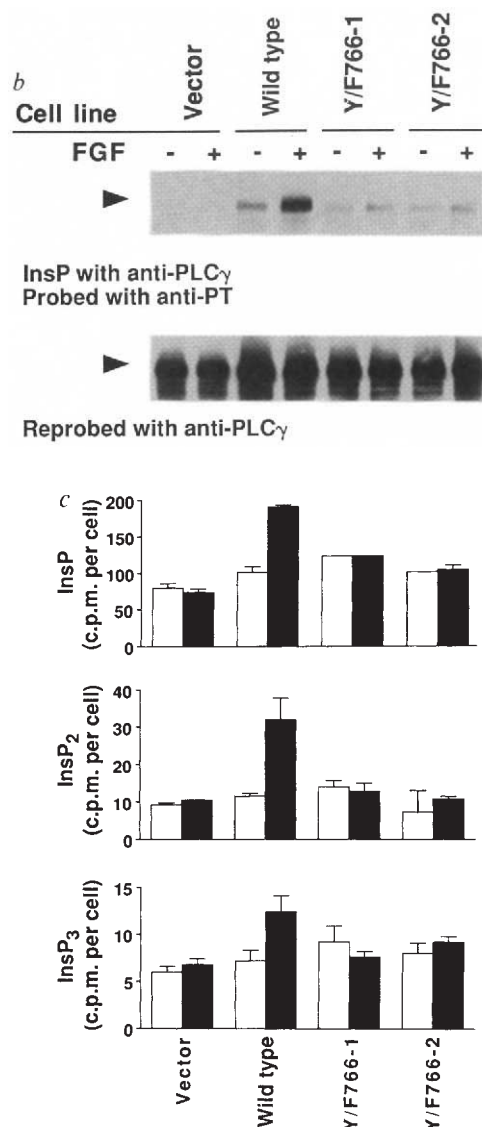


FIG. 2 A mutant FGF receptor with a single point mutation replacing Tyr 766 with phenylalanine (Y/F766) fails to undergo ligand-stimulated association with PLC γ and is defective in mediating the tyrosine phosphorylation and activation of PLC γ *in vivo*. **a**, Failure of the Y/F766 FGF receptor to undergo FGF-stimulated association with PLC γ . Rat L6 myoblasts were transfected with cDNA encoding the wild-type FGF receptor or the Y/F766 mutant FGF receptor (two independent clones, Y/F766-1 and Y/F766-2). To test for the presence of ligand-stimulated association of PLC γ with these receptors, receptor immunoprecipitates from both untreated (-) and FGF-treated (+) cells were probed by immunoblot using an antibody against PLC γ . Arrowhead in the upper panel indicates PLC γ co-immunoprecipitated with the FGF receptor. The blot was reprobed with antibody against the FGF receptor (anti-FR); arrowhead in the lower panel indicates immunoprecipitated FGF receptor. **b**, Mutant FGF receptors defective in PLC γ association are also defective in mediating tyrosine phosphorylation of PLC γ . Anti-PLC γ immunoprecipitates made from unstimulated (-) and FGF-stimulated (+) cells were probed with an antiphosphotyrosine antibody (anti-PT). Arrowhead in the upper panel indicates tyrosine-phosphorylated PLC γ . The blot was reprobed with anti-PLC; the arrowhead in the lower panel indicates immunoprecipitated PLC γ . **c**, Ligand-activated mutant FGF receptors do not mediate increased PtdIns turnover. Open columns, Inositol phosphate (InsP) accumulation in unstimulated cells; solid columns, inositol phosphate accumulation in FGF-stimulated cells. Error bars indicate s.e.m. of triplicate samples.

METHODS. The FGF receptor mutant (Y/F766) was constructed using the cDNA for the two-immunoglobulin-domain form of the human FGF receptor 1 as the template for site-directed mutagenesis with an oligonucleotide containing a single T/A change that encoded the Y/F766 mutation; ACAGGTCCAGGAAGTCTCTGGTTG (ref. 14). Both the wild-type and the mutant cDNA were then subcloned into a pSV7d expression vector and cotransfected with pSV7d-NEO into rat L6 myoblasts. After G-418 selection, clones expressing mutant or wild-type FGF receptors were identified by antiphosphotyrosine immunoblot or receptor immunoblot. For receptor association experiments, subconfluent cultures of L6 cell transfectants (2–4 15-cm plates) were either left unstimulated or were stimulated with basic FGF (50 ng ml⁻¹) for 10 min. After stimulation, cells were lysed in 1% Triton X-100 lysis buffer (1 ml per plate) containing 20 mM Tris, pH 8.0, 137 mM NaCl 10% glycerol, 2 mM EDTA, 1 mM PMSF, aprotinin (0.15 U ml⁻¹), leupeptin (1 μg ml⁻¹) and 1 mM vanadate for 10 min at 4 °C. Lysates were then incubated overnight with antiFR. Immune complexes were collected on



protein A-Sepharose, washed three times with 1% Triton lysis buffer and once with 10 mM Tris, pH 7.4, and analysed by 7% PAGE. Immunoprecipitated protein was transferred to nitrocellulose and subsequently probed with anti-PLC antibody. Experiments to detect tyrosine phosphorylation of PLC γ were done similarly, except that anti-PLC immunoprecipitates were probed with anti-PT. For PtdIns turnover assays, cells in 6-well plates were incubated in Q-medium (DMEM, 20 mM HEPES, pH 7.4, 1 μg ml⁻¹ insulin, 5 μg ml⁻¹ transferrin and 0.5 mg ml⁻¹ BSA) with 10 μCi ml⁻¹ [2-³H(N)]myoinositol for 24 h. After labelling, cells were incubated for an additional 15 min in Q-medium with 20 mM LiCl and then treated with basic FGF (50 ng ml⁻¹) or vehicle for 30 min. At the end of 30 min, cells were extracted with acid (10% HClO₄, 0.5 ml per well). Neutralized extracts were applied to BioRad 1-X8 AG columns (formate form) and free inositol phosphates were eluted in a formate step gradient.

to be phosphorylated more efficiently by the mutant receptor, and at least two additional phosphoproteins were detected after stimulation of the mutant receptor that were not detected after stimulation of the wild-type receptor (Fig. 4b, open arrows). The increased baseline autophosphorylation and the increased phosphorylation of substrates after stimulation of the mutant

receptors may be the result of higher receptor expression, but small differences in the kinase activity between the wild-type and mutant receptors cannot be ruled out. The increased mobility of the ligand-stimulated mutant receptor compared with the wild-type receptor might be secondary to the loss of the Tyr 766 autophosphorylation site or, alternatively, to the loss of phos-

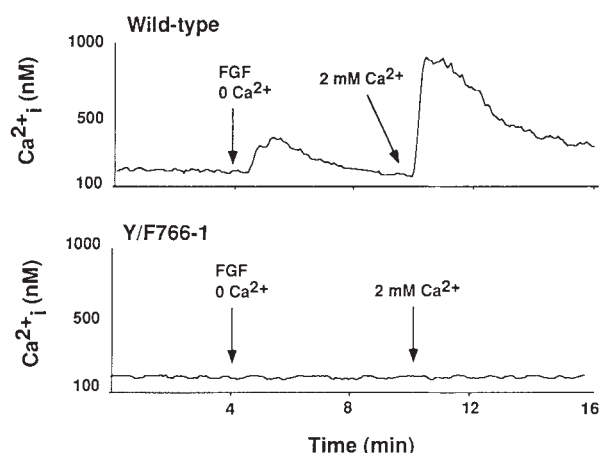


FIG. 3 The Y/F766 mutant FGF receptor does not mobilize Ca^{2+} either from intracellular stores or from the extracellular space in response to FGF. To assess Ca^{2+} mobilization from intracellular stores, basic FGF (5 ng ml^{-1}) was added to L6 myoblasts expressing the wild-type FGF receptor (upper panel) or the Y/F766 FGF receptor (lower panel) in Ca^{2+} -free buffer containing 10 mM EGTA (left arrow). After 10 min the buffer was changed to Ca^{2+} -containing buffer without EGTA (right arrow) to assess the contribution of Ca^{2+} entry to calcium mobilization by the wild-type and Y/F766 mutant FGF receptors.

METHODS. Measurement of intracellular Ca^{2+} was performed with the fluorescent probe Fura-2 as described¹⁷. Intracellular Ca^{2+} concentrations were calculated by comparing the ratios of fluorescence at 340/380 nm obtained at maximal intracellular Ca^{2+} (achieved by addition of $10 \mu\text{M}$ 4Br-A23187) and minimal intracellular Ca^{2+} (achieved by addition of 20 mM EGTA).

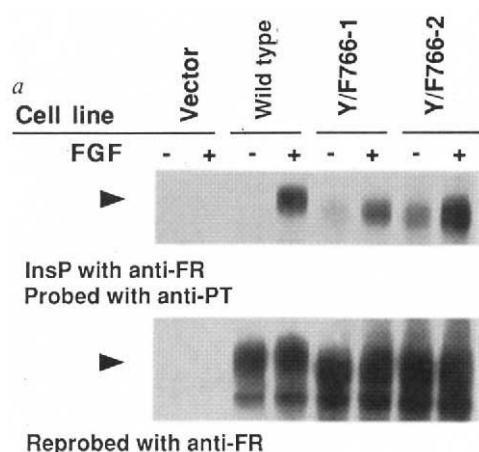
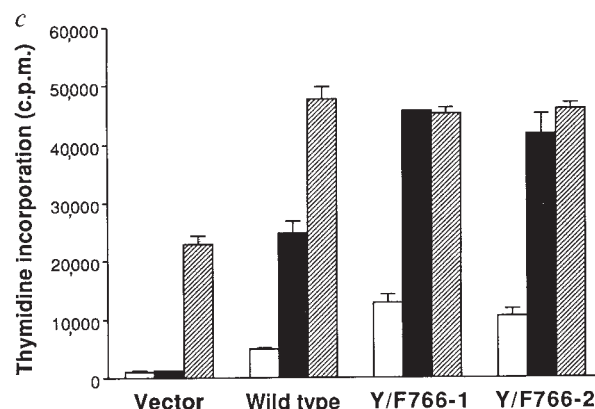
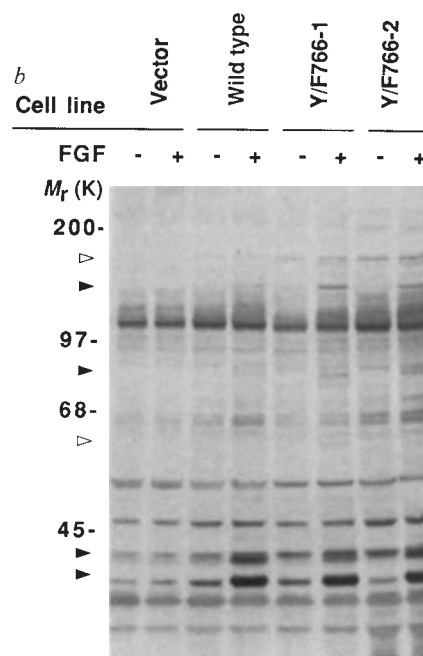


FIG. 4 The Y/F766 FGF receptor mutant is an active kinase that mediates several functions of the wild-type receptor *in vivo*. *a*, The Y/F766 FGF receptor expressed in L6 myoblasts is autophosphorylated after stimulation with FGF. Anti-FR immunoprecipitates from unstimulated (-) and FGF-stimulated cells (+) were probed with anti-PT. Arrowhead in the upper panel indicates autophosphorylated FGF receptor. The blot was reprobed with anti-FR; the arrowhead in the lower panel indicates immunoprecipitated FGF receptor. *b*, Besides autophosphorylation, the Y/F766 mutant mediates ligand-dependent tyrosine phosphorylation of other cellular proteins. Lysates from the L6 cell transfectants were assayed by immunoblot with anti-PT. Closed arrowheads indicate tyrosine phosphoproteins detected after stimulation of either the wild-type or Y/F766 FGF receptor (M_r 145K, 90K, 42K and 35K). Open arrowheads indicate two additional substrates that are detected after FGF stimulation of the Y/F766 receptor (M_r 160K and 55K). *c*, The Y/F766 mutant mediates DNA synthesis in response to FGF stimulation. [^3H]thymidine incorporation was measured in the L6 myoblast transfectants after stimulation with FGF (25 ng ml^{-1}). Open bars, unstimulated; solid bars, FGF-stimulated; hatched bars, serum-stimulated (10% FCS). Error bars indicate s.e.m. of triplicate samples.

METHODS. Lysates for the receptor autophosphorylation experiments and the anti-PT immunoblots were prepared as described in the legend to Fig. 2. For thymidine incorporation assays, cells were plated at equal densities on 24-well culture culture dishes. On day 3 the medium was changed to Q-medium. On day 4 the cells were stimulated for 18 h with the appropriate agent in Q-medium. After stimulation, cells were incubated with [^3H]thymidine ($1 \mu\text{Ci ml}^{-1}$) for 1 h at 37°C . Cells were then washed three times with ice-cold 5% trichloroacetic acid and solubilized for liquid scintillation counting with 0.25 N NaOH in 1% SDS.



phorylation secondary to defective activation of an intracellular serine/threonine kinase. Phosphorylation of tyrosine kinase receptors by serine/threonine kinases is thought to have a negative regulatory effect and might also explain an increase in the kinase activity of the mutant receptor^{18,19}.

Given the proposed importance of PtdIns turnover and Ca²⁺ mobilization in growth factor-stimulated processes and the profound effect FGF treatment of cells has on these pathways, we were surprised to find that FGF stimulated mitogenesis in cells expressing the Y/F766 mutant as well or better than in cells expressing the wild-type receptor. DNA synthesis increased significantly in all cell lines tested (Fig. 4c) and cell numbers increased in proportion to DNA synthesis (data not shown).

PLC γ SH2 domains can bind to Tyr 992 in the C-terminal tail of the epidermal growth factor (EGF) receptor, and a C-terminal tail of a truncated EGF receptor which is also mutated at Tyr 992 still phosphorylates PLC γ but does not activate PtdIns turnover^{20,21}. Also, truncated EGF receptors lacking multiple potential autophosphorylation sites, including Tyr 992, do not associate with or phosphorylate PLC γ , are defective in EGF-mediated receptor downregulation, and can transform cells^{22–25}. The colony-stimulating factor (CSF-1) receptor can mediate mitogenesis in fibroblasts without phosphorylating or activating PLC γ (ref. 26). Overexpression of PLC γ gives increased PtdIns turnover in response to FGF or PDGF but does not enhance mitogenesis in response to either growth factor, suggesting that PtdIns turnover is not limiting in the mitogenic response to FGF or PDGF^{27,28}. Our data demonstrate that increased PtdIns turnover and intracellular calcium mobilization are not required for mitogenesis in response to FGF in L6 myoblasts. Thus other signalling pathways must be involved in mitogenesis by the FGF receptor.

If not required for mitogenesis in response to FGF, what other cellular responses might be triggered by PtdIns turnover? Recent studies suggest that the activation of PLC γ might mediate chemotaxis or cell-shape changes²⁹. Others indicate that PtdIns turnover in response to FGF might mediate cellular differentiation during early embryonic pattern formation³⁰. If the Y/F766 mutant does indeed have a selective signalling defect, it should be useful in establishing the role of FGF-mediated PtdIns turnover in chemotaxis, cell differentiation and other non-mitogenic cellular responses. □

Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis

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STIMULATION of growth factor receptors with tyrosine kinase activity is followed by rapid receptor dimerization, tyrosine autophosphorylation and phosphorylation of signalling molecules such as phospholipase C γ (PLC γ) and the *ras* GTPase-activating protein^{1,2}. PLC γ and GTPase-activating protein bind to specific tyrosine-phosphorylated regions in growth factor receptors^{3–9} through their *src*-homologous SH2 domains^{7,8,10,11}. Growth factor-induced tyrosine phosphorylation of PLC γ is essential for stimulation of phosphatidylinositol hydrolysis *in vitro*¹² and *in vivo*¹³. We have shown that a short phosphorylated peptide containing tyrosine at position 766 from a conserved region^{14–18} of the fibroblast growth factor (FGF) receptor is a binding site for the SH2 domain of PLC γ (ref. 8). Here we show that an FGF receptor point mutant in which Tyr 766 is replaced by a phenylalanine residue (Y766F) is unable to associate with and tyrosine-phosphorylate PLC γ or to stimulate hydrolysis of phosphatidylinositol. Nevertheless, the Y766F FGF receptor mutant can be autophosphorylated, and can phosphorylate several cellular proteins and stimulate DNA synthesis. Our data show that phosphorylation of the conserved Tyr 766 of the FGF receptor is essential for phosphorylation of PLC γ and for hydrolysis of phosphatidylinositol, but that elimination of this hydrolysis does not affect FGF-induced mitogenesis.

To study the role of Tyr 766 in FGF receptor signalling, we generated transfected cell lines expressing either wild-type or three different FGF receptor mutants. The mutant FGF receptors included a point mutant in which Tyr 766 was replaced by a phenylalanine residue (Y766F), a control mutant in which an adjacent non-phosphorylated Tyr 776 was replaced by a phenylalanine residue (Y776F), and a double mutant in which both Tyr 766 and Tyr 776 were substituted by phenylalanine residues (Y766/776F). Wild-type or mutant FGF receptors were expressed in transfected L6 myoblasts lacking endogenous FGF receptors. Several cell lines expressing each mutant receptor were generated and characterized. These cell lines were treated with acidic FGF, lysed, immunoprecipitated with anti-FGF receptor antibodies and, after SDS-PAGE, immunoblotted with either anti-FGF receptor or anti-phosphotyrosine antibodies (Fig. 1). This experiment shows that, in response to acidic FGF, both wild-type and mutant FGF receptors undergo tyrosine autophosphorylation. We next compared tryptic phosphopeptide maps of wild-type and mutant FGF receptors. Figure 2 shows that the tryptic digest of wild-type FGF receptor contains three phosphopeptides and that phosphopeptide P1 (Fig. 2a), which contain Tyr 766 (ref. 8), is missing from the tryptic digests of FGF receptor mutants Y766F and Y766/776F.

We next examined the capacity of mutant FGF receptors to associate with and tyrosine-phosphorylate PLC γ . Figure 3 shows that only wild-type FGF receptor and the FGF receptor Y776F mutant could associate with (Fig. 3a, b) and mediate tyrosine phosphorylation (Fig. 3c, d) of PLC γ in living cells. In contrast, no tyrosine phosphorylation of PLC γ was detected in cells expressing either Y766F or Y766/776F mutants. As growth factor-induced tyrosine phosphorylation of PLC γ is

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essential for its activation^{12,13}, it was expected that FGF receptor mutant Y766F would not be able to stimulate phosphatidylinositol (PtdIns) hydrolysis. Addition of acidic FGF to L6 myoblasts expressing either wild-type or control Y776F FGF receptor mutant led to a large increase in PtdIns hydrolysis (Fig.

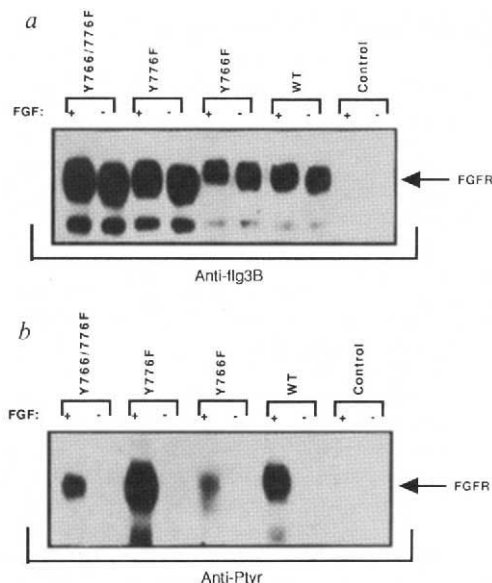


FIG. 1 Acidic FGF-induced tyrosine autophosphorylation of wild-type (WT) and mutant FGF receptors in living cells. Transfected L6 myoblast cell lines expressing either wild-type or FGF receptor (FGFR) mutants were introduced in the presence (+) or absence (-) of acidic FGF, lysed and immunoprecipitated with anti-FGF receptor (anti-flg1A) antibodies followed by SDS-PAGE and immunoblotting with *a*, anti-FGF receptor (anti-flg3B) or *b*, antiphosphotyrosine antibodies (anti-Ptyr).

METHODS. Site-directed mutagenesis was performed according to the manufacturer's protocol (Amersham). A cDNA encoding the full-length human FGF receptor¹⁸ was subcloned in m13MP19 replicative form using *Bam*HI-*Hind*III. Point mutations, which changed Tyr 766 and Tyr 776 either individually or in combination to phenylalanine residue(s) were introduced using oligonucleotides 5'-CCTCAACCAGGAGTTCCTGGACCTGTCCATG-3' (for Y766F mutant), 5'-TGCCCCCTGGACAGTTCCTCCCCAGCTTTC-3' (for Y776F mutant), and 5'-CAGGAGTTCCTGGACCTGTCCATGCCCTGGACAGTTCCTCCCC-3' (for Y766/776F mutant). The cDNA encoding either wild-type or mutant FGF receptors were subcloned into the eukaryotic expression vector pMJ30, under the control of the adenovirus major late promoter and cytomegalovirus enhancer³³. L6 rat myoblasts without endogenous FGF receptors were a subclone selected for high fusion. These cells were transfected with 0.5 μ g pSV2neo and 20 μ g wild-type or mutant FGF receptor expression vectors using calcium phosphate precipitation³⁴. Clones were isolated after 2-3 weeks selection in G418 (Gibco) and screened for expression of FGF receptor by immunoprecipitation with anti-flg1A and immunoblotting with anti-flg3B antibodies. Binding experiments with ¹²⁵I-labelled FGF followed by Scatchard analysis showed that the L6 cells expressing wild-type FGF receptors have $\sim 4.5 \times 10^5$ receptors per cell, Y766F cells have 4.2×10^5 , Y776F cells 5.6×10^5 , and Y766/776F cells $\sim 5.8 \times 10^5$. The dissociation constant for the binding of FGF to all cell lines was $\sim 0.2 \times 10^{-9}$ M. Transfected L6 cells were stimulated with acidic FGF (100 ng ml⁻¹) for 5 min at 37 °C. Cells were washed twice with PBS (Gibco) and scraped into 0.5 ml lysis buffer containing phosphatase inhibitors as described⁵. Lysates were immunoprecipitated with anti-flg1A antibodies immobilized on protein A-Sepharose beads. Immunocomplexes were washed three times with HNTG (20 mM HEPES, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol), 3 $\times$ Laemmli buffer was added and the samples boiled for 5 min. After analysis by SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose membranes and immunoblotted with either anti-flg3B (1:100) or with rabbit polyclonal antiphosphotyrosine antibodies (1:100). The remaining tyrosine-phosphorylated sites on the Y766F mutant were poorly recognized by antiphosphotyrosine antibodies. Blots were treated with ¹²⁵I-labelled protein A and analysed by autoradiography. Rabbit anti-flg3B antiserum was generated against a synthetic peptide from the kinase-insert region (residues 580-586) of human FGF receptor. Anti-flg1A antiserum was generated against a synthetic peptide derived from the C-terminal tail of human FGF receptor (residues 808-822).

4a). As anticipated, acidic FGF was unable to stimulate PtdIns hydrolysis in cells expressing either Y766F or Y766/776F FGF receptor mutants. Hence, elimination of Tyr 766 prevents FGF-induced tyrosine phosphorylation of PLC γ and PtdIns hydrolysis.

Growth factors such as FGF, epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) stimulate association with and tyrosine phosphorylation of PLC γ and subsequent enhancement of PtdIns hydrolysis^{3-9,12,13,19-21}. The role of

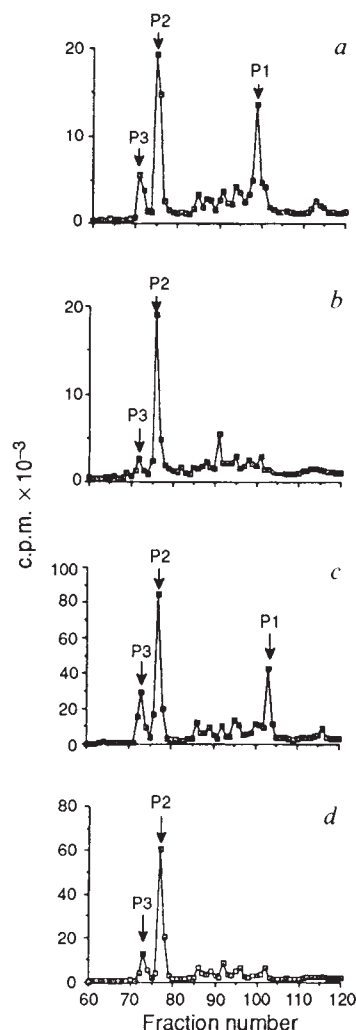


FIG. 2 Comparison of tryptic phosphopeptide maps of wild-type and mutant FGF receptors. Analysis by reverse-phase HPLC of tryptic phosphopeptides of wild-type (a) and FGF receptor mutants Y766F (b), Y776F (c) and Y766/776F (d). Fractions 60-120 contain the previously identified tryptic phosphopeptide P1 (arrow Y766)⁸ and two uncharacterized phosphopeptides, P2 and P3.

METHODS. L6 myoblasts expressing wild-type, Y766F, Y776F or Y766/776F receptor mutants were stimulated with or without acidic FGF (100 ng ml⁻¹) for 5 min at 37 °C, lysed, and the soluble lysates immunoprecipitated with anti-flg1A antibodies. Kinase activity was assayed in 50 ml HNTG containing 5 mM Mn²⁺, 1 μ M unlabelled ATP and 5 μ l [γ -³²P]ATP (6,000 Ci mmol⁻¹) for 10 min at 25 °C. Reaction products were analysed by SDS-PAGE (5-15%) followed by autoradiography. The FGF receptor-specific bands were excised from the SDS gel and digested with trypsin as described⁸. The eluted tryptic peptides were filtered (Millipore) and incubated with monoclonal antiphosphotyrosine antibodies (Oncogene Science) immobilized on beads for 2 h at 4 °C. The phosphotyrosine-containing peptides were eluted with 1 ml 50 mM phenylphosphate in 20 mM HEPES, pH 7.5, and separated on an Aquapore C18 (4.6 by 250 mm) reverse-phase HPLC column as described⁸. Note that the FGF receptor contains two uncharacterized tyrosine-phosphorylation sites (P2 and P3) in addition to Tyr 766 in the P1 phosphopeptide. All three phosphopeptides were also detected on *in vivo* autophosphorylation of FGF receptor in stably or transiently transfected cell lines (data not shown).

growth factor-induced PtdIns hydrolysis as part of signal transduction pathways required for mitogenesis is not clear, however²²⁻²⁶. The fact that FGF did not stimulate PtdIns hydrolysis in untransfected L6 or in L6 expressing the Y766F mutant offered a unique opportunity to dissect the role of PtdIns hydrolysis in FGF-induced mitogenesis. Figure 4b shows that all transfected L6 myoblasts expressing either wild-type or mutant

FGF receptors were mitogenically responsive to acidic FGF with a similar dose dependence. The inability of mutant FGF receptors to stimulate PtdIns hydrolysis did not influence their capacity to mediate a mitogenic signal. We therefore conclude that PtdIns hydrolysis is not required for FGF-induced mitogenesis.

Very little is known about FGF receptor signalling pathways

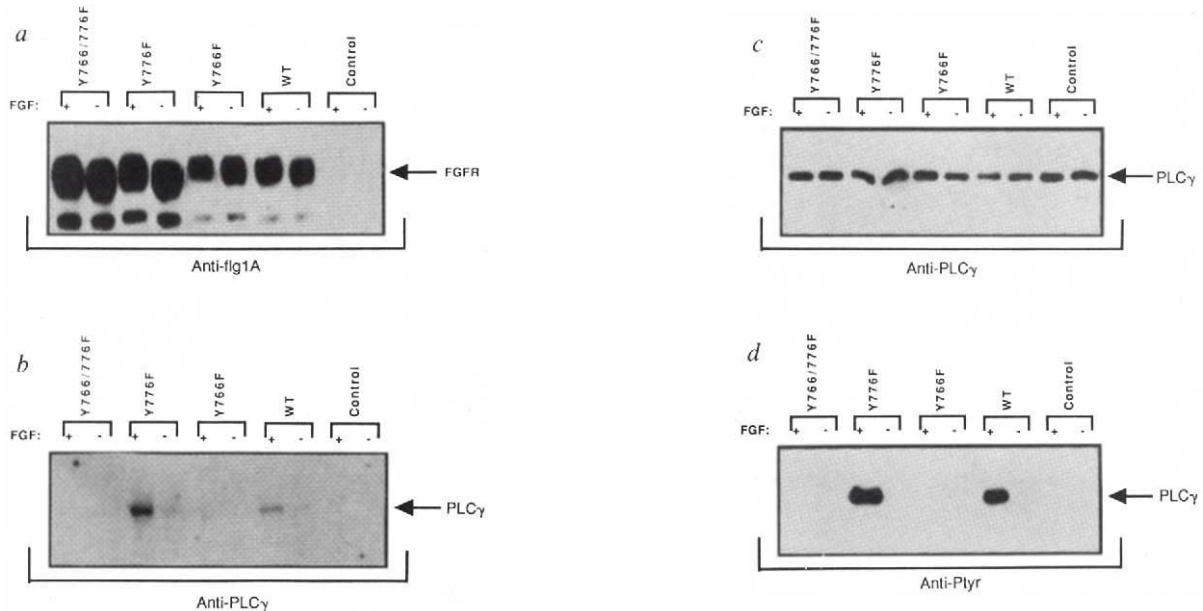


FIG. 3 Point mutation at Tyr 766 of the FGF receptor abolishes association with and tyrosine phosphorylation of PLC γ . L6 myoblasts expressing either wild-type or the three FGF receptor mutants were stimulated with acidic FGF, lysed and immunoprecipitated with anti-FGF receptor antibodies. Samples were analysed by SDS-PAGE and immunoblotted with either anti-FGF-receptor (a) or anti-PLC γ antibodies (b). Transfected L6 myoblasts expressing either wild-type or various FGF receptor mutants were incubated in the presence (+) or (absence (-) of FGF. Lysed cells were immunoprecipitated with anti-PLC γ antibodies followed by SDS-PAGE and immunoblotting with either anti-PLC γ (c) or antiphosphotyrosine antibodies (d).

METHODS. Serum-starved L6 myoblasts or L6 expressing wild-type, Y766F, Y776F or Y766/776F receptor mutants were stimulated with (+) or without (-) FGF (100 ng ml⁻¹) for 5 min at 37 °C, lysed and the soluble lysates were immunoprecipitated with anti-flg3B antibodies. After SDS-PAGE, samples were immunoblotted with either anti-PLC γ or anti-flg1A antibodies. In parallel, lysates from FGF-treated and unstimulated cells were immunoprecipitated with anti-PLC γ antibodies, analysed by SDS-PAGE and immunoblotted with either anti-PLC γ or antiphosphotyrosine antibodies. Rabbit anti-PLC γ antiserum was raised against a synthetic C-terminal peptide of rat PLC γ (residues 1,256-1,274).

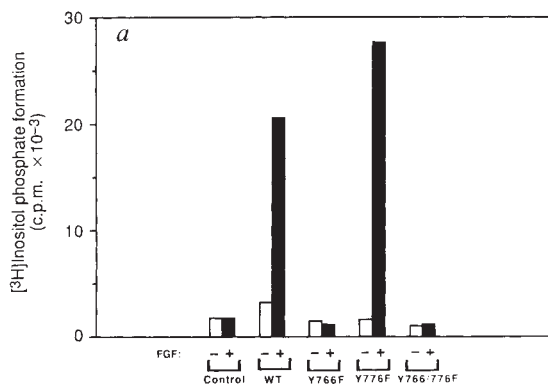
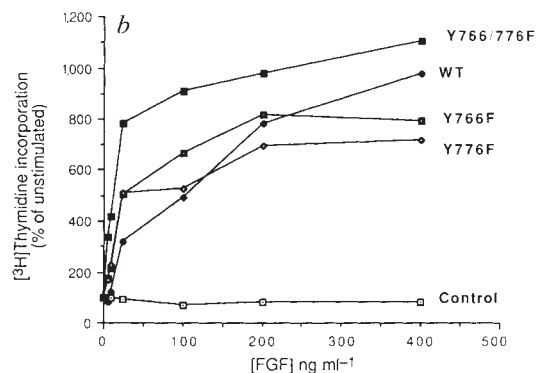


FIG. 4 A point mutation at Tyr 766 of the FGF receptor abolishes FGF-mediated PtdIns hydrolysis without affecting DNA synthesis. a, Inositol phosphate accumulation in unstimulated (open bars) or FGF-stimulated cells (black bars). Data represent the average of triplicate experiments for each cell type. b, Serum-starved cells were stimulated with increasing concentrations of acidic FGF. Average values of percentage increase in thymidine incorporation of three different experiments are presented.

METHODS. Phosphatidylinositol hydrolysis: L6 myoblasts expressing either wild-type or FGF receptor mutants were labelled with [³H]myoinositol (2 μ Ci ml⁻¹) in DMEM containing 0.5% FBS for 24 h and incubated in DMEM containing 20 mM LiCl for 20 min before addition of acidic FGF (100 ng ml⁻¹) for an additional 30 min at 37 °C. Cells were extracted with 5% perchloric



acid and inositol phosphate formation was measured according to published procedures²². Thymidine incorporation: L6 cells were seeded in 96-well plates (2 $\times$ 10⁴ cells per well) and 24 h later the medium was changed to DMEM containing 0.1% FBS. After 48 h serum starvation, the medium was replaced by medium containing FGF or, as a control, 10% FCS. After stimulation for 24 h, cells were incubated with [³H]thymidine (0.5 μ Ci ml⁻¹) for 16 h at 37 °C. Cells were washed with PBS, trypsinized and collected using a PHD Cell Harvester (Cambridge Technologies) and the amount of incorporated [³H]thymidine was quantitated by liquid scintillation counting (LKB). Acidic FGF was also able to stimulate the proliferation of the various transfected L6 cell lines. Similar results were obtained with transfected 3T3 cells expressing endogenous FGF receptors (data not shown).

that may be required for FGF-induced mitogenesis. Signalling molecules such as PtdIns 3-OH kinase or *ras* GTPase-activating protein (GAP) that become associated with many activated growth factor receptors do not seem to interact with the activated FGF receptor (ref. 27, and our unpublished results). Moreover, it is not yet clear whether downstream serine/threonine kinases such as Raf or Map2 kinase are activated on stimulation of FGF receptors. We have shown here that the intrinsic protein tyrosine kinase activity of the Y766F mutant is maintained and that this mutant is able to undergo autophosphorylation on at least two additional uncharacterized tyrosine residues (Fig. 2). It is therefore likely that the interaction between FGF receptor and substrates crucial for FGF-induced mitogenesis is not dependent on autophosphorylation of Tyr 766. Indeed, several proteins are tyrosine-phosphorylated in Y766F cells in response to FGF stimulation (data not shown).

Our main conclusions are that elimination of Tyr 766 of the FGF receptor abolishes PLC γ association with the receptor, PLC γ tyrosine phosphorylation and PtdIns hydrolysis and that PtdIns hydrolysis is not required for FGF-induced mitogenesis. This is also consistent with studies showing that colony-stimulating factor 1, insulin-like growth factor 1 and insulin are all able to stimulate DNA synthesis although these growth factors do not stimulate PLC γ phosphorylation and PtdIns hydrolysis^{1,28}. In general then, PtdIns hydrolysis may not be required for mitogenesis triggered by activation of receptors with tyrosine kinase activity. Yet growth factors such as FGF, PDGF and EGF are clearly able to stimulate PtdIns hydrolysis. Hence, FGF-induced PtdIns hydrolysis could be crucial for the regulation of other non-mitogenic responses mediated by FGF. For example, FGF-induced PtdIns hydrolysis could be involved in the regulation of cellular differentiation. In early amphibian embryogenesis, FGF induces the formation of ventral mesoderm^{29,30} and PtdIns hydrolysis is crucial for its development³¹, which suggests a mechanism by which FGF may control mesoderm formation during amphibian embryogenesis. FGF-induced PtdIns hydrolysis might also be involved in the control of cell shape and morphology. Phosphatidylinositol(4,5)-biphosphate is able to interact specifically with the actin-binding protein profilin³², which may increase the amount of monomeric actin available for polymerization and so influence cell shape and motility. Hence the Y766F FGF receptor mutant could be a powerful tool for dissecting the role of PtdIns hydrolysis in cellular responses mediated by FGF. □

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Structure of an SH2 domain of the p85 α subunit of phosphatidylinositol-3-OH kinase

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RECEPTOR protein-tyrosine kinases, through phosphorylation of specific tyrosine residues, generate high-affinity binding sites which direct assembly of multienzyme signalling complexes^{1,2}. Many of these signalling proteins, including phospholipase C γ , GTPase-activating protein and phosphatidylinositol-3-OH kinase, contain *src*-homology 2 (SH2) domains, which bind with high affinity and specificity to tyrosine-phosphorylated sequences^{3,4}. The critical role played by SH2 domains in signalling has been highlighted by recent studies showing that mutation of specific phosphorylation sites on the platelet-derived growth factor receptor impair its association with phosphatidylinositol-3-OH kinase, preventing growth factor-induced mitogenesis^{5,6}. Here we report the solution structure of an isolated SH2 domain from the 85K regulatory subunit of phosphatidylinositol-3-OH kinase, determined using multidimensional nuclear magnetic resonance spectroscopy. The structure is characterized by a central region of β -sheet flanked by two α -helices, with a highly flexible loop close to functionally important residues previously identified by site-directed mutagenesis^{7,8}.

Phosphatidylinositol-3-OH kinase (PtdIns-3-OH kinase) is a heterodimeric protein, consisting of a regulatory subunit of *M_r* 85K and a 110K catalytic subunit (p85 α and p110, respectively)^{9,10}. Through its SH2 domains, p85 α binds to activated protein-tyrosine kinases and acts as an adapter protein, mediating the recruitment of the catalytic p110 subunit to the plasma membrane. The amino-terminal SH2 domain, included within residues 314–431 of bovine p85 α , was expressed as a glutathione S-transferase fusion protein¹¹. Compared to SH2 domains as defined by sequence homology^{3,12} the 118 amino-acid fragment used in this study contains a total of ~20 amino acids in N- and carboxy-terminal extensions. These extra residues were included because a recombinant fragment corresponding to the consensus domain displayed at least a 10-fold reduced affinity for binding to a phosphorylated peptide corresponding to the sequence surrounding Tyr 751 of the platelet-derived growth factor (PDGF) receptor. The longer recombinant SH2 domain displayed enhanced binding ($K_d = 3 \times 10^{-9}$ M, data not shown) and solubility, suggesting that the N- and C-terminal extensions contribute to the correct folding and stability of the SH2 domain. The affinity-purified fusion protein was cleaved using thrombin, a procedure that resulted in the addition of two amino acids (Gly, Ser) at the N terminus of the SH2 domain-containing fragment. ¹⁵N-labelled material was purified in the same way from bacteria grown in minimal medium¹³ containing ¹⁵NH₄Cl

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as the only nitrogen source. Both preparations resulted in homogeneous protein as judged by SDS-polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC) analysis (not shown).

This material was studied by nuclear magnetic resonance (NMR) spectroscopy. Sequential assignment of amino-acid residue spin systems was made after comparison of strips from within the amide region of three-dimensional ^1H nuclear Overhauser enhancement ^{15}N - ^1H heteronuclear multiple quantum

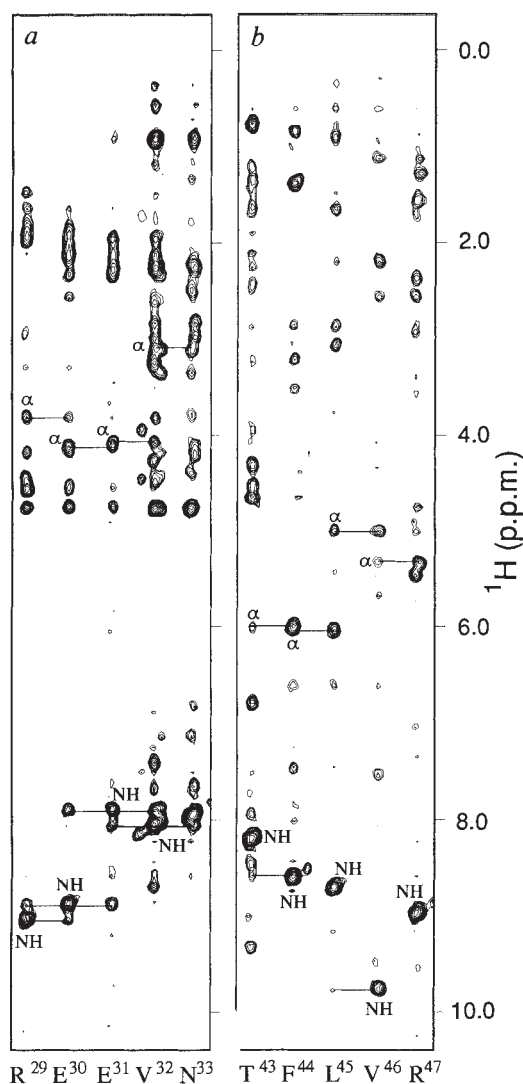


FIG. 1 Strips taken from the amide region of selected ^{15}N planes of a three-dimensional ^{15}N - ^1H NOESY-HMQC spectrum, (mixing time 150 ms) in sequence order showing: a, a region of α -helix 1 (residues 29-33); b, a region of β -strand 1 (residues 43-47). Autocorrelation ^{15}N - ^1H peaks (NH) and intra-residue ^{15}NH - $\text{C}\alpha\text{H}$ NOEs (α) were assigned after comparison with the corresponding regions of a three-dimensional ^{15}N - ^1H HOHAHA-HMQC spectrum. Horizontal lines indicate the presence of sequential NOEs used to substantiate sequential assignments. ^{15}N -enriched SH2 samples were prepared as previously described²⁴ except that the bacterial expression was conducted in appropriately transformed *Escherichia coli* BL21 cells grown in minimal medium¹³ containing $^{15}\text{NH}_4\text{Cl}$ as the only nitrogen source. Two- and three-dimensional NMR spectra were recorded at 500 MHz or 600 MHz with various sample concentrations (1.2 mM and 3.5 mM for two-dimensional ^1H NMR and 1 mM for ^{15}N - ^1H three-dimensional NMR, in 80 mM sodium phosphate pH 5.8) at a temperature of 35 °C. Sequential assignments were made on the basis of three-dimensional ^{15}N - ^1H NOESY-HMQC spectra whereas side-chain assignments were obtained using a combination of two-dimensional ^1H double-quantum-filtered COSY, ^1H HOHAHA and ^1H NOESY spectra recorded on samples dissolved in D_2O , in combination with the three-dimensional spectra.

TABLE 1 Structural statistics

	(SA)	(SA) _r
r.m.s. deviations from experimental distance restraints ($\text{\AA}$)*		
All (1,187)	0.047 ± 0.002	0.040
Interproton distances		
Intraresidue (267)	0.045 ± 0.004	0.037
Sequential (354)	0.045 ± 0.004	0.039
Inter-residue short-range ($1 < i-j \leq 4$) (150)	0.047 ± 0.005	0.036
Inter-residue long-range ($ i-j > 4$) (362)	0.050 ± 0.004	0.044
H-bonds (54)†	0.062 ± 0.009	0.046
r.m.s. deviations from experimental dihedral restraints (deg)*‡	0.48 ± 0.13	0.30
Deviations from idealized covalent geometry		
Bonds ($\text{\AA}$)	0.009 ± 0.0	0.009
Angles (deg)	2.161 ± 0.007	2.439
Impropers (deg)§	0.970 ± 0.013	0.950
F_{NOE} (kJ mol^{-1})	426.5 ± 41.0	303.7
F_{repel} (kJ mol^{-1})¶	236.1 ± 22.1	190.2
E_{VDW} (kJ mol^{-1})#	$-1,131.5 \pm 52.5$	-972.0

The statistics of structural calculations from Fig. 2 are shown here. The notation of the structures is as follows: (SA) are the 22 refined simulated annealing structures; (SA)_r is the restrained minimized mean structure, where the mean structure was obtained by averaging the coordinates of the individual (SA) structures best-fitted to each other. The number of terms for the various restraints is given in parentheses.

* None of the structures exhibit distance violations greater than 0.5 $\text{\AA}$ or dihedral violations greater than 3°.

† Each hydrogen-bond is characterized by two distance restraints: $d_{\text{NH}\cdots\text{O}}$, 2.1-2.3 $\text{\AA}$ and $d_{\text{N}\cdots\text{O}}$, 2.1-3.3 $\text{\AA}$. All hydrogen-bonding restraints involve slowly exchanging backbone amide protons and were only included after they could be unambiguously assigned following initial structure calculations.

‡ The torsion angle restraints comprise 30ϕ angles.

§ The improper torsion restraints serve to maintain planarity and chirality.

|| The value of the square-well NOE (F_{NOE}) is calculated with a force constant of $210 \text{ kJ mol}^{-1} \text{\AA}^{-2}$ (ref. 22).

¶ The value of the quartic van der Waals repulsion term F_{rep} is calculated with a force constant of $17 \text{ kJ mol}^{-1} \text{\AA}^{-4}$ (ref. 22) with the hard sphere van der Waals radius set to 0.8 times their standard values.

The van der Waals energy E_{VDW} is calculated with the CHARMM²³ empirical energy function and is not included in the target function for simulated annealing or restrained minimization.

coherence (NOESY-HMQC) and ^1H homonuclear Hartmann-Hahn ^{15}N - ^1H -HMQC (HOHAHA-HMQC) spectra^{14,15}. Representative strips selected from the regions of the first α -helix and first β -strand are shown in Fig. 1 along with nuclear Overhauser effects (NOEs) used in the sequential assignment. Analysis of the short-range connectivities¹⁶ and $\text{C}\alpha\text{H}$ chemical shifts¹⁷ indicated the pattern of secondary structural elements of the domain to be $\alpha\beta\beta\beta\beta\alpha\beta\beta$. Figure 2 shows a best-fit superposition of the 22 final structures and a histogram illustrating the number of NOE distance constraints and r.m.s. deviation by residue number. Residues 1 to 16 of the recombinant protein failed to provide structurally significant NMR data and lie outside the region of strong sequence similarity which defines the SH2 domain. They have therefore been excluded from the structure calculations and are not shown in the figure. Three-dimensional structures were calculated from the experimental constraints with the program X-PLOR^{18,19}. Initial structures with approximately correct three-dimensional folds were generated using a dynamic simulated annealing (SA) protocol^{19,20} starting from randomly generated backbone configurations with extended side chains. These structures were refined using an efficient SA protocol^{21,22}.

A summary of the structural statistics for the individual SA structures and for the minimized average structure is presented in Table 1. The overall atomic r.m.s. difference between the

FIG. 2 *a*, Superposition of 22 final structures of the p85 α N-terminal SH2 domain shown in stereoview. *b*, Histogram of the number of NOE distance constraints (open bars) and r.m.s. deviation values (closed bars) plotted against residue number for the structures shown in *a*. Also shown in *b* are the relative positions within the peptide chain of β -strands (numbered 1–7) and α -helices (labelled helix 1 and helix 2). Three categories of distance constraints were used in calculations corresponding to estimated intensities of NOE crosspeaks: weak ≤ 5.0 Å, medium ≤ 3.3 Å, strong ≤ 2.8 Å. The appropriate corrections for centre averaging were made to distances involving methyl and methylene protons²⁵. $^3J_{\text{NH}\alpha}$ spin-spin coupling constants were determined by line shape fitting traces of peaks from a two-dimensional ^{15}N - ^1H HMQC-J experiment²⁶. Slowly exchanging amide protons were identified by their continued observation in two-dimensional ^{15}N - ^1H HMQC experiments at least 2 h after dissolution in D_2O . The final structure calculations were based on 1,481 interproton distance constraints of which 1,133 were assessed as structurally significant by the variable target function program DIANA²⁷. These restraints include 267 intra-residue, 354 sequential ($|i-j|=1$), 150 short-range ($1 < |i-j| \leq 4$) and 362 long-range ($|i-j| > 4$) constraints. Additional restraints used in structure calculations included 54 distance constraints for 27 backbone hydrogen-bonds and 30 ϕ dihedral angle constraints. A total of 83 refined simulated annealing (SA) structures converged from 100 random coil configurations. Of these, 22 SA structures were selected on the basis of agreement with the experimental data, with the cut-off taken at $F_{\text{NOE}} < 490 \text{ kJ mol}^{-1}$. These are shown superposed with best-fit on the backbone atoms of residues 17–49, 56–102 and 110–118. A mean structure was calculated from the 22 individual best-fitted structures by coordinate averaging. The covalent geometry of the mean structure was regularized by restrained minimization as in the last stage of the refinement. This restrained minimized average structure (SA), satisfies the experimental constraint data as well as any of the individual SA structures. The resulting structures were visualized on a Silicon Graphics 4D/20 graphics system with the program Insight (Biosym Technologies).

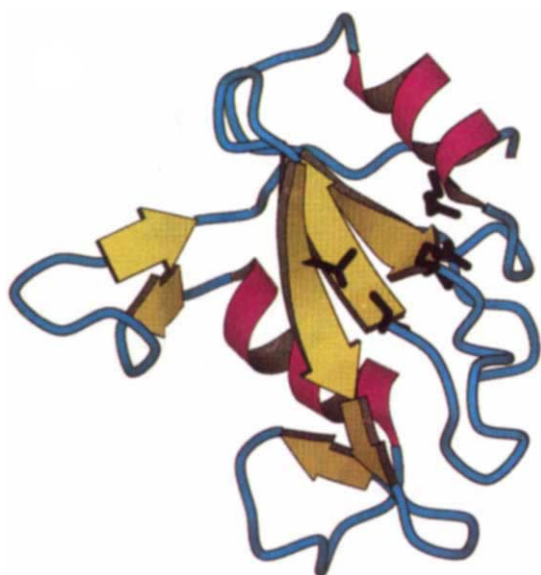
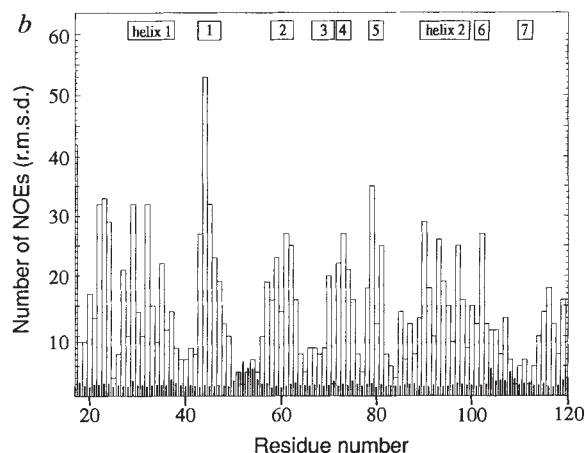
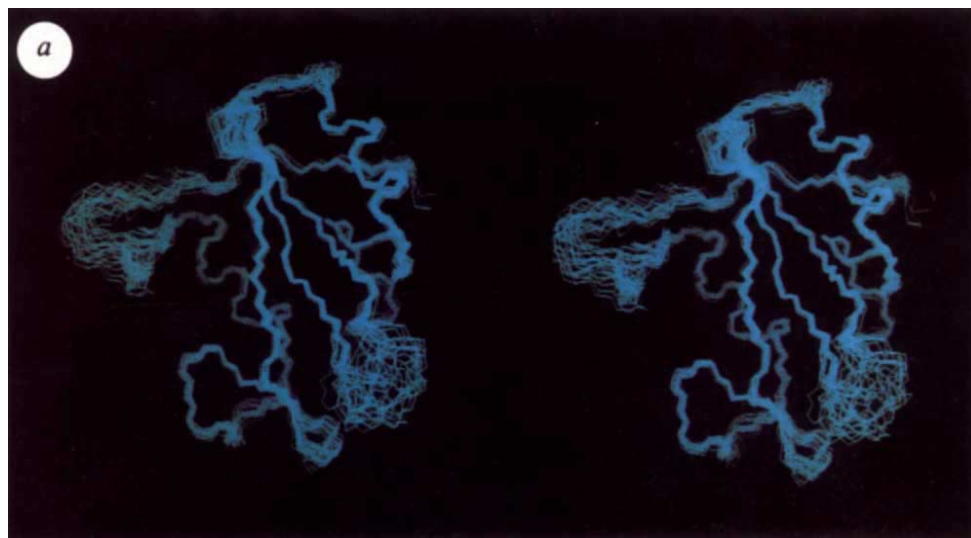


FIG. 3 Schematic representation of the average structure of p85 α N-terminal SH2 domain, showing the relative orientation of the β -sheets and α -helices. Also shown are the side chains of four strongly conserved residues Arg 29, Arg 47 (contained within the conserved motif FLVR), Thr 58 and Leu 69 indicating the possible location of the phosphotyrosine binding site. The diagram was produced using the program MOLSCRIPT²⁸.

individual structures and the mean coordinate positions is 0.48 ± 0.08 Å for the backbone atoms (residues 17–49, 56–102 and 110–118) and 1.17 ± 0.13 Å for all heavy atoms (residues 17–50 and 55–120) indicating that the structures are of medium resolution. Residues excluded from these calculations were considered to be disordered on the basis of the available NMR data. All of the chosen structures satisfy the NMR constraints within the experimental error, with no constraint violations over 0.5 Å and no torsion angle violations greater than 3°. Deviations from idealized geometry are small, and the negative value of $-1,131 \text{ kJ mol}^{-1}$ for the Lennard-Jones energy (which is not used in the target function for the SA calculations) indicates that nonbonded contacts are good²³.

The structure can be described as a central three-stranded antiparallel β -sheet with a kink at the end of the third strand (Lys 71) preceding a short two-stranded antiparallel β -sheet. Two amphipathic helices pack onto either side of the central β -sheet, mainly through hydrophobic interactions. A second short two-stranded antiparallel β -sheet near the C terminus folds back to lie over one face of the central β -sheet, placing the N and C termini of the molecule (as defined by sequence homology) near each other on one side of the molecule. These regions of secondary structure are linked by a number of tight turns and mobile loops such as that between strands 1 and 2 of the central β -sheet. Note the good agreement of recent secondary structure prediction studies¹² (G.P. *et al.*, manuscript in preparation) with our structure.

The structure reported here allows the results of mutagenesis and sequence comparison studies to be interpreted more fully.

When 67 SH2 domain sequences are aligned to maximize homology¹², 14 residues are either absolutely conserved or represented by only two alternative amino acids. Of these 14, 10 have side chains that are buried within the molecule (residues 22, 23, 36, 42, 44–46, 72, 87 and 93) and are therefore likely to be important for maintaining structural integrity. The remaining four strongly conserved residues Arg 29, Arg 47, Thr 58 and Leu 69 (corresponding to His 294 in *crk*) cluster specifically at the surface of the molecule, as shown schematically in Fig. 3. Arg 47 and Leu 69 represent positions implicated as pivotal to phosphotyrosine binding activity in mutagenesis studies of *crk* and *abl* SH2 domains^{7,8}. These studies also showed that Ser 173 and Ser 175 of *abl* SH2 are important for binding activity, yet they are not highly conserved residues. The corresponding positions in our p85 α SH2 are Ala 49 and Thr 51, respectively, which are found within the highly mobile loop joining β -strands 1 and 2. In the light of this, we propose that the region between the N-terminal end of α -helix 1 and the central β -sheet represents the phosphotyrosine binding site, with the possibility that the mobile loop comprising residues 49–55 folds inward to hold the bound peptide in place. This would explain the functional importance of residues Ala 49 and Thr 51 despite the low degree of conservation of these positions throughout SH2 domains. Studies of SH2 domains complexed with phosphotyrosine peptides should cast more light on the nature of this important interaction. □

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Nested expression domains of four homeobox genes in developing rostral brain

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INSIGHT into the genetic control of the identity of specific regions along the body axis of vertebrates¹ has resulted primarily from the study of vertebrate homologues of regulatory genes operating in the *Drosophila* trunk², but little is known about the development of most anterior regions of the body either in flies^{3,4} or vertebrates. Three *Drosophila* genes have been identified that are important in controlling the development of the head^{5–8}, two of which, *empty spiracles*⁵ and *orthodenticle*⁸, have been cloned and shown to contain a homeobox^{9–11}. We previously cloned and characterized *Emx1* and *Emx2*, two mouse genes related to *empty spiracles* that are expressed in restricted regions of the developing forebrain, including the presumptive cerebral cortex and olfactory bulbs¹². Here we report the identification of *Otx1* and *Otx2*, which are related to *orthodenticle*^{7,8}. We have compared the expression domains of the four genes in the developing rostral brain of mouse embryos at a developmental stage, day 10 post coitum, when they are all expressed. *Otx2* is expressed in every dorsal and most ventral regions of telencephalon, diencephalon and mesencephalon. The *Otx1* expression domain is similar to that of *Otx2*, but contained within it. The *Emx2* expression domain is comprised of dorsal telencephalon and small diencephalic regions, both dorsally and ventrally. Finally, *Emx1* expression is exclusively confined to the dorsal telencephalon. Thus at the time when regional specification of major brain regions takes place, the expression domains of the

four genes seem to be continuous regions contained within each other in the sequence $Emx1 < Emx2 < Otx1 < Otx2$.

The exon-intron organization of the homeobox regions of *Otx1* and *Otx2* are shown in Fig. 1a and b, respectively. *Otx1* and *Otx2* homeodomains differ at three and two amino-acid residues from the *orthodenticle* (*otd*) homeodomain, respectively (Fig. 1c). In particular, the three homeodomains share the lysine residue at position 9 of the third recognition helix also present in *Drosophila bicoid* (*bcd*)¹⁰ and frog *gooseoid* (*gsc*)¹³ genes.

We studied the expression of the two genes in early mouse embryos using the DNA probes shown in Fig. 1a, b. We first analysed 7.5-, 8.5-, 9- and 9.75-d.p.c. (days post coitum) mouse embryos in sagittal sections (Fig. 1d–j). *Otx1* is first expressed

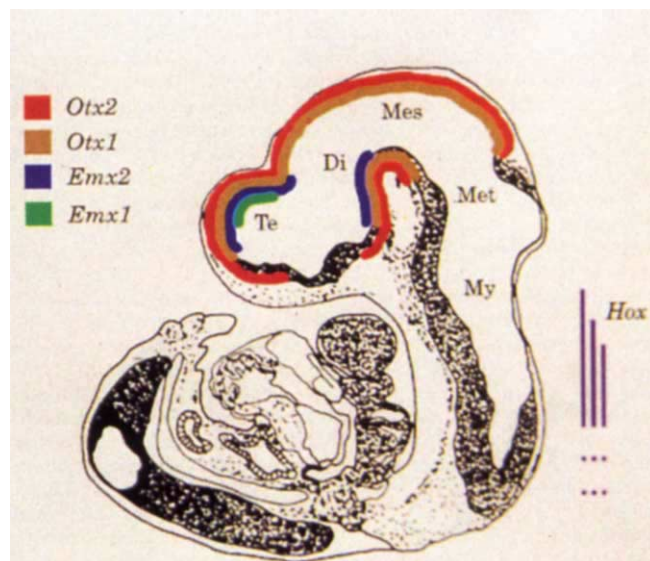


FIG. 4 Summary of the expression domains of the four genes in the developing central nervous system in 10-d.p.c. mouse embryos. Expression of members of the Hox gene family is also indicated. Di, diencephalon; Mes, mesencephalon; Met, metencephalon; My, myelencephalon; Te, telencephalon.

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in a large region of the anterior neural tube of 8.5 d.p.c. embryos (Fig. 1d). Anterior-posterior delimitation of *Otx1* expression in the rostral neural tube is clear in 9 d.p.c. (Fig. 1e) and in 9.75-d.p.c. (Fig. 1f) embryos. Dorsally its expression domain comprises a continuous region, including part of the telencephalon, the diencephalon and the mesencephalon¹⁴. The posterior boundary of this domain coincides with that of the mesencephalon (arrow), but in median sections, such as that shown in Fig. 1f, a strong hybridization signal extends only half way

(open arrow) along the mesencephalon. Ventrally, the *Otx1* expression domain includes contiguous regions of both diencephalon and mesencephalon with sharp anterior and posterior boundaries.

Otx2 is expressed at an earlier developmental stage than *Otx1* as a hybridization signal is already detectable in very anterior regions of 7.5-d.p.c. embryos, including the headfold (Fig. 1g). *Otx2* is also expressed in 8.5- and 9-d.p.c. embryos (Fig. 1h, i), with an expression domain containing that of *Otx1* and including

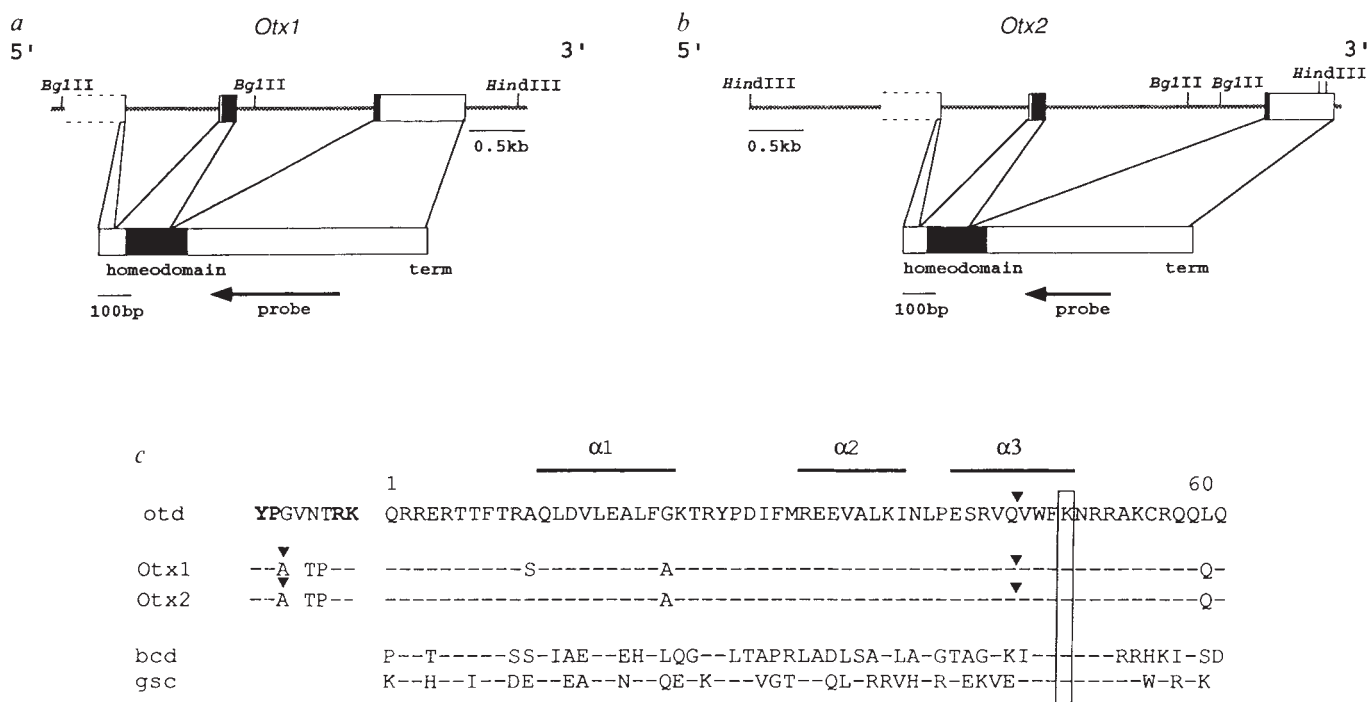
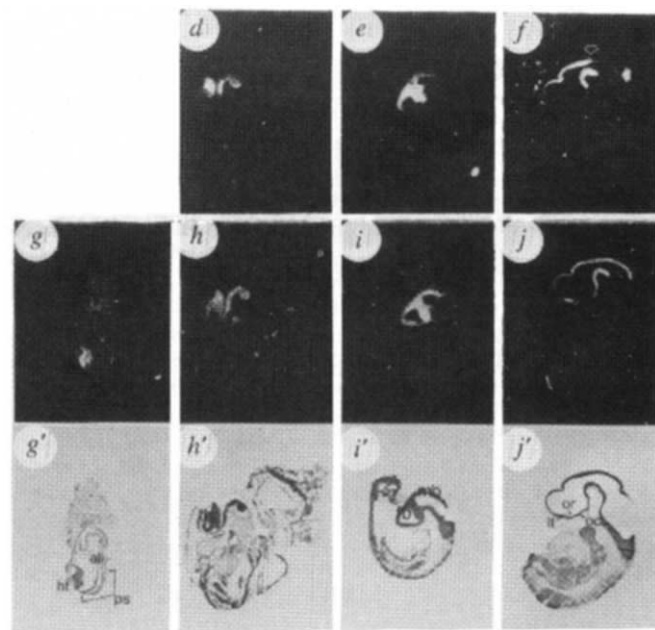


FIG. 1 Structure and expression of *Otx1* and *Otx2*. Cloned complementary DNA region and genomic organization of part of *Otx1* (a) and *Otx2* (b). Boxes indicate exons and a black box indicates the homeodomain. The probe used for *in situ* hybridization experiments is shown below the cDNA scheme. c, Comparison of *Otx1* and *Otx2* homeodomains with *otd*, *bcd* and *gsc* homeodomains (one-letter amino-acid code). Dashes indicate amino-acid identity with *otd*, arrowheads point to splice sites. The three helix motifs of the homeodomain are shown and the lysine residue at position 9 of the third helix is boxed. d-f, *Otx1* expression in sagittal sections of 8.5 d.p.c. (d), 9 d.p.c. (e) and 9.75 d.p.c. (f) mouse embryos; g-j, *Otx2* expression in sagittal sections of 7.5 d.p.c. (g), 8.5 d.p.c. (h), 9 d.p.c. (i) and 9.75 d.p.c. (j) mouse embryos. The solid arrow in f points to the posterior boundary of the dorsal *Otx1* expression domain, the open arrow to the posterior boundary of the strong *Otx1* hybridization signal. Hybridization probes are shown in a and b. A bright field exactly corresponding to a given dark field is indicated by a prime affix; for example, the section corresponding to the dark field in g is indicated as g'. Abbreviations: all, allantois; fb, forebrain; hf, headfold; lt, lamina terminalis; mb, midbrain; oc, optic chiasma; or, optic recess; ps, primitive streak.

METHODS. A cDNA library prepared from 11-day mouse embryos (Clontech) was screened under low-stringency conditions²² with a short *otd* genomic sequence including the homeobox. This *otd* genomic region was obtained by amplification using the polymerase chain reaction (PCR) of *Drosophila* total DNA with two synthetic oligonucleotide primers derived from the *otd* sequence⁵. Analysis of *otd* amplified regions provided evidence for the presence of the intron shown in c. Two classes of homologous cDNA clones, termed *Otx1* and *Otx2*, were found. Using these cDNA clones as probes we screened in turn a genomic library constructed in cosmids and compared corresponding regions in cDNA and genomic clones. *Otx1* and *Otx2* encode proteins that share with *otd* two residues, Arg-Lys, upstream from the homeodomain and the general structure. All three contain a relatively short N-terminal domain upstream from the homeodomain and a long C-terminus downstream from it. An intron is present in *Otx1* and *Otx2* immediately upstream from the homeodomain, as is often the case for homeobox genes²³. The exon upstream from this intron ends in all three genes with the motif Tyr-Pro-Ala/Gly, possibly a divergent version of the conserved



homeopentapeptide Ile/Phe-Tyr-Pro-Tro-Met present in several homeotic genes of *Drosophila* and in most vertebrate genes belonging to the Hox clusters²³ (Fig. 1c). An additional intron is present in the three genes within the homeobox at identical positions, namely between residues 46 and 47 of the homeodomain. For mouse embryos, day 0.5 p.c. was assumed to begin at the middle of the day of vaginal plugging. *In situ* hybridizations were carried out as described²⁴.

the entire forebrain. Anterior-posterior delimitation of the *Otx2* expression is clear in 9.75-d.p.c. embryos (Fig. 1j). Dorsally, it includes the entire telencephalon, the diencephalon and the mesencephalon. The anterior portion of this domain includes lamina terminalis and presumptive striatum, whereas the posterior boundary coincides with that of the mesencephalon. Ventrally, the *Otx2* expression domain includes contiguous regions of diencephalon and mesencephalon with an anterior boundary just posterior to the optic chiasma.

We then compared the expression domains of *Otx1* and *Otx2* to those of *Emx1* and *Emx2* at a developmental stage, 10 and 10.25 d.p.c., when the four genes are all expressed in the developing neural tube. In sagittal sections of 10-d.p.c. embryos (Fig. 2a-d), the four expression domains are clearly defined as they are in median (Fig. 2e-h) and paramedian (Fig. 2i-l) sagittal sections of 10.25-d.p.c. embryos. The *Emx1* expression domain (Fig. 2a, e, i) includes the dorsal telencephalon with a posterior boundary probably coinciding with that between presumptive diencephalon and telencephalon. *Emx2* is expressed (Fig. 2b, f, j) in dorsal neuroectoderm with an anterior boundary slightly anterior to that of *Emx1* and a posterior boundary well within the roof of presumptive diencephalon. Ventral expression in the floor of diencephalon is also evident at this developmental stage. A few extracephalic localizations of *Emx2* expression¹² are also observable in 10.25-d.p.c. embryos (Fig. 2f-j) in the coelomic epithelium covering the mesonephric column, as well as the final part of the mesenteric attachment (arrow).

Otx1 and *Otx2* expression domains at this stage are essentially those of 9.75-d.p.c. brain. Both dorsally and ventrally, the *Otx1* expression domain (Fig. 2c, g, k) contains the *Emx2* domain. It covers a continuous region including part of the telencephalon,

the diencephalon and the mesencephalon, with an anterior boundary roughly coincident with that of *Emx2*. In paramedian sections (Fig. 2k) the posterior boundary of this domain coincides with that of the mesencephalon, even if in more median sections (Fig. 2c, g) a strong hybridization signal extends only half-way along the mesencephalon. A subdivision of early mesencephalon in two neuromeres has been proposed¹⁵. The *Otx2* expression domain (Fig. 2d, h, l) contains the *Otx1* domain both dorsally and ventrally. It covers practically the entire fore- and midbrain; only the regions of optic chiasma and optic recess are excluded.

Frontal and transverse sections (Fig. 3) of 10-d.p.c. embryos confirm the relative localization of the expression domains of the four genes in dorsal and ventral brain regions, defining a rostral brain as opposed to deuterencephalon¹⁴ and spinal cord. In the first two transverse sections (Fig. 3f-i) no expression of either *Emx1* or *Emx2* is detectable, but in more central sections (Fig. 3j-m) expression of all four genes is detectable and its extension can be compared.

We have compared the expression domains of four homoeobox genes in the developing brain of mouse embryos at a developmental stage when the four genes are all expressed (Figs. 2, 3). Their expression domains are continuous regions contained within each other in the sequence *Emx1* < *Emx2* < *Otx1* < *Otx2* (Fig. 4, page 687). The first appearance of the four

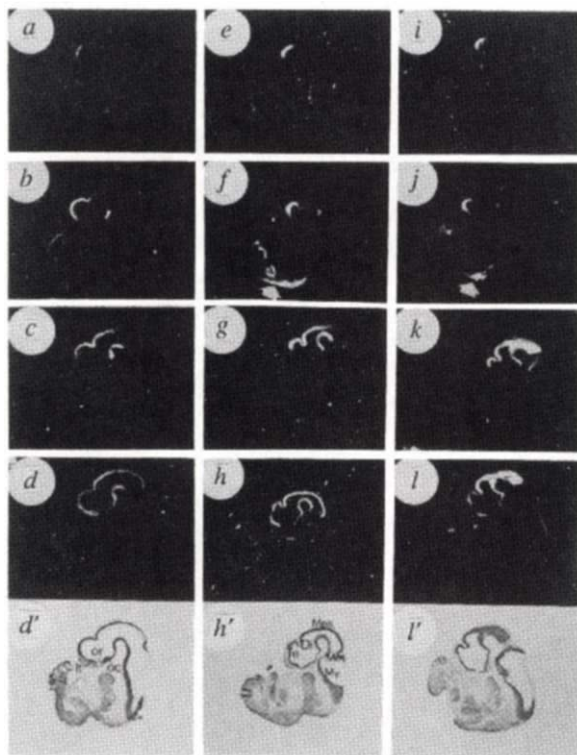


FIG. 2 Gene expression in sagittal sections of 10 d.p.c. (a-d) and 10.25 d.p.c. (e-l) mouse embryos. a, e and i, Hybridization with *Emx1*; b, f and j, hybridization with *Emx2*; c, g and k, hybridization with *Otx1*; d, h and l, hybridization with *Otx2*. Sections i-l are more paramedian. Arrows in f, j point to *Emx2* expression spots in the trunk. Abbreviations as in Fig. 1; Di, diencephalon; Mes, mesencephalon; Met, metencephalon; My, myelencephalon; Te, telencephalon.

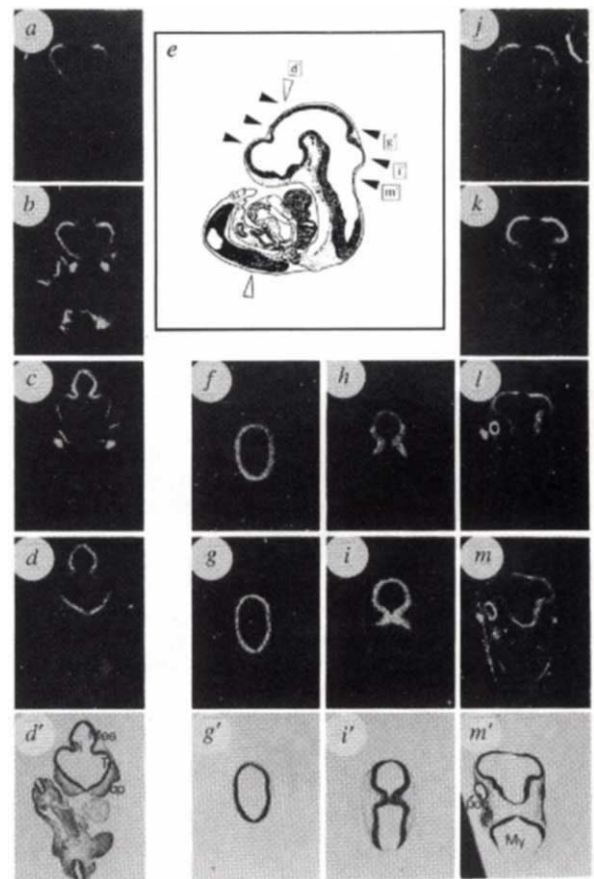


FIG. 3 *Emx1*, *Emx2*, *Otx1* and *Otx2* expression in frontal (a-d) and transverse (f-m) sections of 10-d.p.c. mouse embryos. a and j, Hybridization with *Emx1*; b and k, hybridization with *Emx2*; c, f, h and l, hybridization with *Otx1*; d, g, i and m, hybridization with *Otx2*; e, scheme of frontal and transverse sections. Arrows in b and c point to *Emx2* and *Otx1* expression in ectodermal localizations around the olfactory placodes. Arrows in l and m point to *Otx1* and *Otx2* expression in optic cups. Abbreviations as in Figs 1 and 2; op, olfactory placode; poc, presumptive optic cup.

genes is also sequential: *Otx2* is expressed first (7.5 d.p.c.), followed by *Otx1* and *Emx2* (8.5 d.p.c.), and finally by *Emx1* (9.5 d.p.c.). Several homeobox genes are believed to control cell identity with a regional or segmental pattern both in flies and vertebrates (refs 1, 16–19, and see ref. 20 for a review). The four genes may have a role in establishing and/or signalling the limits and identity of the various embryonic brain regions^{14,15}. Although it is not yet possible to assign the restricted domains to morphological segments, these domains may correspond to some of the neuromeric segmentation already proposed^{14,15}. The specification of the various regions of the rostral brain therefore seems to be a discrete progressive process with its centre in the dorsal telencephalon.

The cephalization process is thought to have occurred independently in the evolutionary lineages leading to insects and vertebrates^{14,20,21}. Nevertheless, *empty-spiracles*-related and *orthodenticle*-related genes are expressed in anterior cephalic regions both in flies and mammals. Taken with the conservation of the Hox system, it is remarkable that several of the different systems potentially involved in specifying anterior regional variation in the mammalian nervous system correspond to similar *Drosophila* processes. It will be interesting to investigate the actual role played by genes of this type and the evolution of their expression patterns. □

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Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation

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INCREASING evidence indicates that the integrin family of cell adhesion receptors can transduce biochemical signals from the extracellular matrix to the cell interior to modulate cell growth and differentiation¹. We have shown that integrin/ligand interactions can trigger tyrosine phosphorylation of a protein of *M_r* 120,000 (pp120), so it is possible that signal transduction by integrins might involve activation of intracellular protein tyrosine kinases as an early event in cell binding to the extracellular matrix². Here we report that pp120 is identical to the focal adhesion-associated protein tyrosine kinase pp125^{FAK} (refs 3, 4). We show that tyrosine phosphorylation of this protein is modulated both by cell adhesion and transformation by pp60^{v-src}, and that these changes in phosphorylation are correlated with increased pp125^{FAK} tyrosine kinase activity. A model is proposed to relate these findings to the molecular basis of anchorage-independent growth of transformed cells.

Earlier studies showed that tyrosine phosphorylation of pp120 can be induced by plating NIH3T3 cells on either plasma fibronectin or on anti-integrin antibody, but not on nonspecific substrates such as poly-L-lysine or concanavalin A (ref. 2). These studies excluded the possibility of pp120 being an integrin β 1, vinculin or α -actinin². To see whether pp120 could be identified with a known phosphoprotein, we used specific antibodies to immunoprecipitate candidate proteins from lysates prepared from NIH3T3 cells plated on either polylysine or plasma fibronectin. Immunoprecipitates were then western blotted with

antiphosphotyrosine antibodies to detect any tyrosine phosphorylation. Figure 1 shows the result for three other candidate proteins: two potential v-src protein substrates, p120 (not to be confused with pp120) and pp125^{FAK} (ref. 4), and the Ras GTPase-activating protein (GAP)⁵. Under our conditions (no serum), no tyrosine-phosphorylated GAP or p120 was detected in cells plated either on polylysine or plasma fibronectin (lanes 3–6). But pp125^{FAK} from the cells plated on fibronectin had increased tyrosine phosphorylation compared with pp125^{FAK} from cells plated on polylysine (lanes 7, 8). Furthermore, phosphorylated pp125^{FAK} comigrated with pp120 (ref. 2), the tyrosine phosphorylation of which depends on the cells being plated on plasma fibronectin as detected in total cell lysates (lane 2). These results suggested that pp120 could be identical to the potential v-Src substrate pp125^{FAK}.

We therefore isolated pp120 from NIH3T3 cells grown on plasma fibronectin by affinity chromatography using immobil-

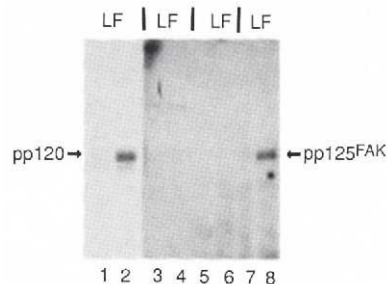


FIG. 1 Cell-adhesion-dependent tyrosine phosphorylation of pp125^{FAK}. Lysates were prepared from NIH3T3 cells plated on dishes that had been coated with poly-L-lysine (lanes L) or plasma fibronectin (lanes F) as described². Lysates containing equal amounts of total cell proteins were immunoprecipitated using antibodies against GAP (lanes 3, 4), p120 (lanes 5, 6) and pp125^{FAK} (lanes 7, 8). They were electrophoresed on an SDS-polyacrylamide gel with the same amount of total cell lysates (lanes 1, 2), transferred to a nitrocellulose membrane and probed by antiphosphotyrosine antibody py20 (ICN) as described².

METHODS. Immunoprecipitations were done by incubating lysates with antibodies for 1 h at 4 °C. For monoclonal antibodies, rabbit anti-mouse IgG serum (Sigma) was then added to the mixture and incubated for 1 h at 4 °C. Immune complexes were collected on protein A-Sepharose and washed 4 times in lysis buffer. Precipitates were eluted by boiling for 3 min in SDS sample buffer.

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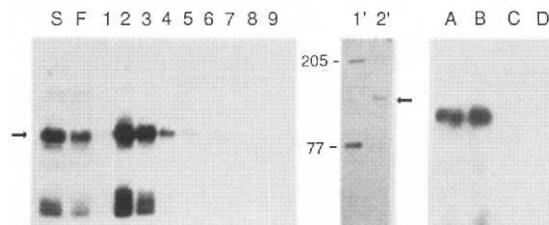
ized antiphosphotyrosine antibody py20 and analysed it by SDS-PAGE followed by western blotting with antiphosphotyrosine antibody (Fig. 2, left panel). Consistent with previous data, pp120 was the principal phosphoprotein detected in the starting material (lane S) and most of it was bound to py20-agarose beads (compare lanes F, flow-through, and S). The peak fractions eluted (lanes 2 and 3) contained pp120 migrating as a distinct band, which accounted for at least 80% of the protein in the peak fractions, as assessed by silver staining after electrophoresis (lane 2'). The pooled peak fractions (lane A) were then immunoprecipitated successively with an anti-serum against pp125^{FAK} (BC3; provided by J. T. Parsons) and analysed by western blotting with py20 (Fig. 2, right panel). The

pp125^{FAK} immunoprecipitation clearly removed all pp120, indicating that pp120 is identical with pp125^{FAK} immunologically. Immunofluorescent staining showed that pp125^{FAK} localizes in focal contacts in cells plated on fibronectin, as did pp120 detected by py20 before (refs 2, 3 and J.-L.G., unpublished data).

The protein pp125^{FAK} was first identified in pp60^{v-src} transformed cells as a potential substrate of the v-Src tyrosine kinase^{3,4}. Molecular cloning and sequencing of its complementary DNA indicated that it contains a tyrosine kinase domain, and it is active as a kinase when expressed in *Escherichia coli*⁴. The identification of pp120 as pp125^{FAK} raises the possibility that it is regulated both by cell adhesion through integrins and by transformation by oncogenes encoding tyrosine kinases. To

FIG. 2 Affinity purification of pp120 by immobilized antiphosphotyrosine antibody (py20) and its identification as pp125^{FAK}. Left panel: lysates from NIH3T3 cells were applied to a py20-agarose column (see Methods). Bound proteins were eluted with buffer containing 5 mM phosphotyrosine, and an aliquot of each fraction (lanes 1–9) with the starting material (S) and the flow-through (F) were analysed by western blotting using py20. The position of pp120 is indicated on the left by an arrow. Middle panel: silver stain of the purified pp120 (arrowed, right) pooled from fractions 2, 3 and 4 (lane 2'). M_r markers (205K and 77K) are in lane 1'. Right panel: to confirm that pp120 is pp125^{FAK}, purified pp120 (lane A) was immunoprecipitated by anti-pp125^{FAK} as described in Fig. 1 (lane B). Unbound materials were precipitated again using the same antibody (lane C) and this was repeated (lane D). Samples were analysed by western blot using py20.

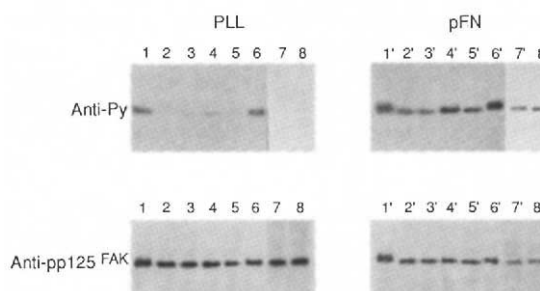
METHODS. Affinity chromatography using py20-agarose (ICN) was adapted from ref. 12. NIH3T3 cells from ten 150-mm plates were lysed in a buffer containing 10 mM imidazole, 0.5 M NaCl, 1% Triton-X100, 0.2 mM vanadate, 0.2 mM PMSF, 2 mM Na₂S₂O₈, pH 7.3, at 4 °C for 30 min (2 ml per plate). Lysates were clarified by centrifugation at 100,000g for 1 h and the supernatant incubated with 1 ml py20-agarose (10 mg antibody per ml Sepharose) for



1 h at 4 °C. The agarose beads were then collected by centrifugation, loaded into a column, and washed extensively with buffer containing 10 mM imidazole, 0.1 M NaCl, 0.1% Triton-X100. Bound proteins were eluted with the same buffer containing 5 mM phosphotyrosine and 0.5-ml fractions collected. Peak fractions containing pp120 were identified by western blotting of aliquots as described for Fig. 1. An aliquot of the pooled peak fractions was concentrated by precipitation with 12% trichloroacetic acid and analysed by SDS-PAGE followed by silver staining.

FIG. 3 Tyrosine phosphorylation of pp125^{FAK} in 3T3 cells expressing different Src proteins. pp125^{FAK} was immunoprecipitated from various cell lines plated on poly-L-lysine (PLL; left panel) or plasma fibronectin (pFN; right panel) as described in Fig. 1 legend. Duplicate samples were electrophoresed on SDS-Polyacrylamide gels, transferred to nitrocellulose membranes and probed with either anti-phosphotyrosine antibody (top) or anti-pp125^{FAK} antibody (bottom). The different forms of Src expressed in 3T3 cells are as follows: v-Src (lanes 1, 1'), non-myristylated c-Src (lanes 2, 2'), kinase-defective c-Src (lanes 3, 3'), c-Src (lanes 4, 4'), c-SrcF416 (lanes 5, 5'), and c-SrcF527 (lanes 6, 6'). Also shown is tyrosine phosphorylation of pp125^{FAK} in untransfected 3T3 cells (lanes 7, 7') and Ras-transformed 3T3 cells (lanes 8, 8').

METHODS. Cell lines NIH(pMvsrc/foc/EP)A4, NIH(pCLN/pSV2neo/MC)C, NIH(pCr295/pSV2neo/cos)J, NIH(pMcsrc/foc)B, NIH(pcsrc416/pSV2neo/MC)A, NIH(pcsrc527/foc/EP)B1 expressing v-Src, wild-type and mutant c-Src have been described⁶⁻⁸. Ras-3T3 cells were transformed with plasmid pEJ6.6 (ref. 13). All procedures were as described in Fig. 1 legend, with the following modifications. Rabbit antiphosphotyrosine serum was generated



as in ref. 14 and used for western blotting instead of py20, and the enhanced chemiluminescence reaction (Amersham) with horseradish peroxidase-conjugated goat-anti-rabbit IgG was used instead of ¹²⁵I-conjugated secondary antibodies.

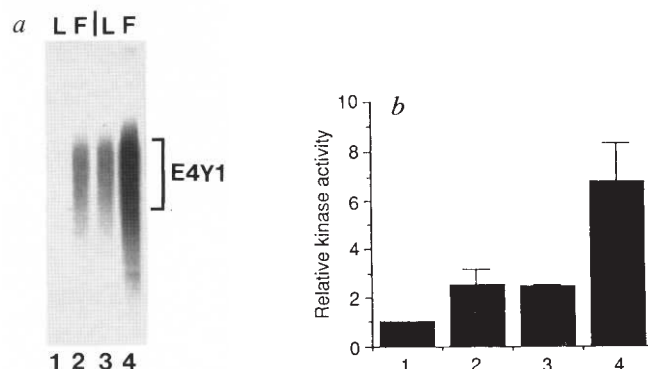


FIG. 4 Tyrosine phosphorylation of pp125^{FAK} activates its tyrosine kinase activity. pp125^{FAK} was immunoprecipitated from 3T3 cells (lanes 1, 2) and v-src-transformed 3T3 cells (lanes 3, 4) that had been plated on either poly-L-lysine (lanes 1, 3) or plasma fibronectin (lanes 2, 4). Immune complex tyrosine kinase assays were done in a buffer containing [γ -³²P]ATP and an exogenous substrate poly(Glu-Tyr) (4:1). Radiophosphorylated substrates were subjected to SDS-PAGE and autoradiography (a). The region containing the labelled substrate (bracketed, E4Y1) was excised and quantified by scintillation counting. The average and standard deviation of relative kinase activities were obtained from three independent experiments (b). Relative activities were normalized to pp125^{FAK} from 3T3 cells on polylysine.

METHODS. Immunoprecipitates were washed three times in lysis buffer and once in kinase buffer (10 mM MnCl₂, 50 mM Tris, pH 7.4). The immune complex was then incubated with 30 μ l kinase buffer, 10 μ Ci [γ -³²P]ATP (3,000 Ci mmol⁻¹, NEN) and 10 μ g poly(Glu-Tyr) (4:1; Sigma) for 20 min at room temperature. Reactions were stopped by adding SDS sample buffer and analysed by electrophoresis and autoradiography.

test this, pp125^{FAK} was immunoprecipitated from NIH3T3 cell lines overexpressing v-src, c-src and mutant src oncoproteins⁶⁻⁸, and analysed by western blotting using both anti-pp125^{FAK} and anti-phosphotyrosine antibodies (Fig. 3). For cells plated on polylysine (left panel), pp125^{FAK} from 3T3 cells expressing v-src gave a large (~10-fold) increase in tyrosine phosphorylation (lane 1) relative to that in pp125^{FAK} from untransformed 3T3 cells (lane 7, Fig. 1). There was similar increase in cells expressing activated c-Src with a Tyr 527 → Phe mutation (c-Src(F527); ref. 7) (Fig. 3, lane 6). Wild-type c-Src had much less effect: 16-fold overexpression of c-Src elevated pp125^{FAK} tyrosine phosphorylation only about 2-fold (lane 4). This effect was abrogated by a mutation that prevents myristylation (lane 2), by a Lys 295 → Arg mutation that eliminates kinase activity (lane 3), or by a Tyr 416 → Phe mutation (lane 5). The wild-type and mutant c-Src proteins were all comparably expressed (data not shown). For cells plated on plasma fibronectin (right panel), pp125^{FAK} was tyrosine phosphorylated in all cell lines (lanes 1' to 6'). Characteristic mobility retardations of pp125^{FAK} from cells expressing v-Src and c-Src(F527) were observed⁴. It appears that cell-adhesion-dependent tyrosine phosphorylation of pp125^{FAK} (compare panels pFN and PLL) and Src-induced phosphorylation are additive and perhaps independent of each other (compare lanes 1' and 1 with 2' and 2, for example).

Taken together, our results indicate that induction of pp125^{FAK} tyrosine phosphorylation in cells lacking integrin-mediated adhesion (plated on polylysine) correlates with induction of transformation by different forms of Src. But, increased phosphorylation is not a consequence of cell transformation because Ras-transformed 3T3 cells do not show increased pp125^{FAK} phosphorylation when plated on polylysine (Fig. 3, lane 8). Therefore it is possible that tyrosine phosphorylation of pp125^{FAK} might contribute to cellular transformation by oncogenes encoding tyrosine kinases such as v-Src.

If pp125^{FAK} mediates signals initiated by integrin-dependent cell adhesion in a way that can be overridden by src transformation, its functions should be regulated by its increased tyrosine phosphorylation during cell adhesion or transformation. To test this, pp125^{FAK} was immunoprecipitated from 3T3 or v-src 3T3 cells that had been plated on polylysine or on plasma fibronectin. Half of each immunoprecipitate was analysed by western blot with anti-pp125^{FAK} serum to verify that each sample contained the same amount of pp125^{FAK} (not shown). The other halves were assayed *in vitro* for kinase activity (Fig. 4). The activity associated with pp125^{FAK} from 3T3 cells plated on plasma fibronectin was about 2.5 times that from cells plated on polylysine. On both substrates the kinase activity associated with pp125^{FAK} from v-src transformed 3T3 cells was higher than from parental 3T3 cells. Thus the kinase activity associated with pp125^{FAK} is upregulated by tyrosine phosphorylation induced by either integrin/fibronectin interaction (compare columns 1 and 2) or v-Src transformation (compare columns 3, 4 with 1, 2); the combined effects are multiplicative.

Anchorage-independent growth of transformed cells is one of the hallmarks of cellular transformation in which the normal requirement for substrata attachment to proliferate is reduced or lost^{9,10}. It is possible that extracellular matrix molecules support normal cell growth by transmission of signals to the cell interior by integrins. In transformed cells such signalling pathways might be constitutively activated independently of cell adhesion, thereby conferring anchorage-independent growth. Results from this and previous studies^{2,3,11} suggest that pp125^{FAK} is in the integrin signalling pathway. We have shown here that it can also be tyrosine-phosphorylated and activated in v-src transformed cells even when cells are plated on polylysine so that integrin/ligand interactions are lacking. Furthermore, tyrosine phosphorylation and activation of pp125^{FAK} induced by Src variants correlates with their abilities to transform cells.

Experiments are in progress to define the sites of tyrosine phosphorylation to determine whether the activation of

pp125^{FAK} cell adhesion and v-src transformation results from the same mechanism. In blood platelets pp125^{FAK} is hyperphosphorylated and activated in an integrin-dependent manner (L. Lipfert *et al.*, personal communication); the same phosphorylation sites may be involved in these cases. It is important to identify the *in vivo* substrates of pp125^{FAK} as these are likely to participate in the normal signalling pathways of integrins. Besides activating pp125^{FAK} directly, v-Src might short-circuit the normal pathway for integrin-initiated signal transduction by phosphorylating pp125^{FAK} substrates that are critical for downstream events. □

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Fast sequencing of oligosaccharides using arrays of enzymes

Christopher Edge, Raj Parekh, Thomas Rademacher, Mark Wormald and Raymond Dwek

A new strategy for sequencing oligosaccharides involves generating fragments at 'stop points' along the molecule using enzyme matrices.

THE relevance of oligosaccharides in defining the structure and function of glycoconjugates is becoming increasingly evident¹. These important molecules have now been shown in several instances to act as precise ligands in specific recognition events, to modulate important cellular activities and to affect glycoprotein function. There is therefore an anticipated need for rapid, easy and automated approaches to the isolation and structural analysis of oligosaccharides.

Primary sequence analysis of oligosaccharides is complicated by the number of parameters that need to be determined in order to define completely the sequence of an oligosaccharide (for example, monosaccharide sequence, anomericity of each glycosidic bond, branching and substitution patterns). The principal chemical and physical methods of determining oligosaccharide sequence are nuclear magnetic resonance, various forms of mass spectrometry, permethylation analysis and enzymatic analysis using exoglycosidases² and, to a lesser extent, endoglycosidases. Other useful techniques include sequential lectin chromatography and the use of anti-carbohydrate monoclonal antibodies.

Endo- and exoglycosidases

Exoglycosidases are enzymes that cleave only the monosaccharides that occupy the nonreducing terminal positions of oligosaccharides. In contrast, endoglycosidases such as endo H and endo F cleave the monosaccharides that occupy the reducing terminal position (only one) of an oligosaccharide.

Exoglycosidases can have a broad specificity (that is, cleave hexose $\alpha 1 \rightarrow 2$, hexose $\alpha 1 \rightarrow 3$, hexose $\alpha 1 \rightarrow 4$, hexose $\alpha 1 \rightarrow 6$) or have restricted specificity (that is, only cleave hexose $\alpha 1 \rightarrow 2$ linkages). In general, exoglycosidases are anomer specific (that is, α or β). The ability of an exoglycosidase to remove a terminal monosaccharide can also depend on what the penultimate residue is, or even depend on substitutions not at the actual site of cleavage. When all of these cleavage rules are known for a particular exoglycosidase it becomes a powerful reagent for determining the sequence of a multibranched oligosaccharide.

Sequential exoglycosidase analysis

Enzymatic analysis of oligosaccharide structure is generally performed by incubating an oligosaccharide structure with an

exoglycosidase of well-defined specificity, identifying the product chromatographically, and so determining and measuring the loss of monosaccharide(s), if any, induced by the enzyme. With this information, a decision is made as to which exoglycosidase to use next (based usually on preliminary monosaccharide composition information and/or a knowledge of the oligosaccharide biosynthesis pathway in the source from which the oligosaccharide was obtained), and the iterative process is continued until the oligosaccharide has been 'sequenced', or until no further useful information can be obtained using the available exoglycosidases². There are three principal disadvantages of this 'sequential' process. (1) It is very time consuming, requiring repeated purification, incubation and chromatography. (2) There is inevitable progressive sample loss as the steps are repeated. (3) The application of glycosidases is *ad hoc*. It is necessary to guess the order in which enzymes should be applied and considerable effort can be wasted trying the wrong enzymes. Further, the process is irreversible so that mistakes in the order of enzyme application cannot be rectified. (4) The process does not make consistent use of negative information (that is, if an oligosaccharide contains only terminal galactose, it will be nonreactive towards mannosidases etcetera).

Enzyme-array analysis fingerprints

A recent report³ describes an approach, referred to as the reagent array analysis method (RAAM), which allows exoglycosidases to be used for oligosaccharide structural analysis in a way that avoids some of the drawbacks of the sequential approach. The RAAM, schematically summarized in the figure, involves dividing an oligosaccharide solution into equal aliquots. Each aliquot is incubated with a precisely defined mixture of exoglycosidases, the products of each incubation are combined, and a single analysis is performed on the pool of products. The set of mixtures of exoglycosidases is referred to as the 'reagent array'. In essence, a mixture of exoglycosidases is used to generate a fragment of the oligosaccharide, and by omitting one or more different exoglycosidase(s) from each mixture, different fragments of the oligosaccharide are generated. In each enzyme mix digestion will continue until a linkage is reached that is resistant to all the exoglycosidases present

in that mix. Full use is therefore made of all positive data (the exoglycosidases hydrolyse linkages up to the 'stop' point) and negative data (the exoglycosidases do not hydrolyse linkages beyond the 'stop' point). By labelling the original oligosaccharide at the single reducing terminus, products retaining the original reducing terminus are readily distinguished from released monosaccharides. Chromatographic separation of the pool of products generates a pattern of fragments that is in effect a 'fingerprint' of that oligosaccharide generated by the specific array used. This fingerprint is characterized by the elution position and relative signal intensity of each fragment. This fingerprint can then be matched either to a database of experimentally determined fingerprints of standard oligosaccharides or, more informatively, against a computer-generated database of 'theoretical' fingerprints. In the latter case, very large numbers of oligosaccharides can be 'screened' for a match against the experimental data, thus broadening the applicability of the method. For the computer-generated database approach to be successful, the separation system used to generate the fingerprint must be predictable to a reasonable accuracy (for example, gel filtration, mass spectrometry are suitable) and provide sequence-independent signal intensity (for example, labelling systems based on reduction of the reducing terminus are suitable). A complete application of this approach has now been provided³.

Matching the fingerprints

It must be possible to predict the fingerprint from either theoretical considerations or known empirical rules. This requires that both the specificities of the exoglycosidases used in an array should be well defined and that the separation means by which fragments are resolved should be predictable. Ideally, therefore, the separation means should be based on an intrinsic property of oligosaccharides, such as mass or hydrodynamic volume.

Two key practical considerations in this approach are the experimental accuracy with which the elution position and relative intensity of each fragment can be determined, and the ability of exoglycosidases to retain independent activity in mixtures. Even allowing for significant errors in the measurement of fragment position and intensity³,

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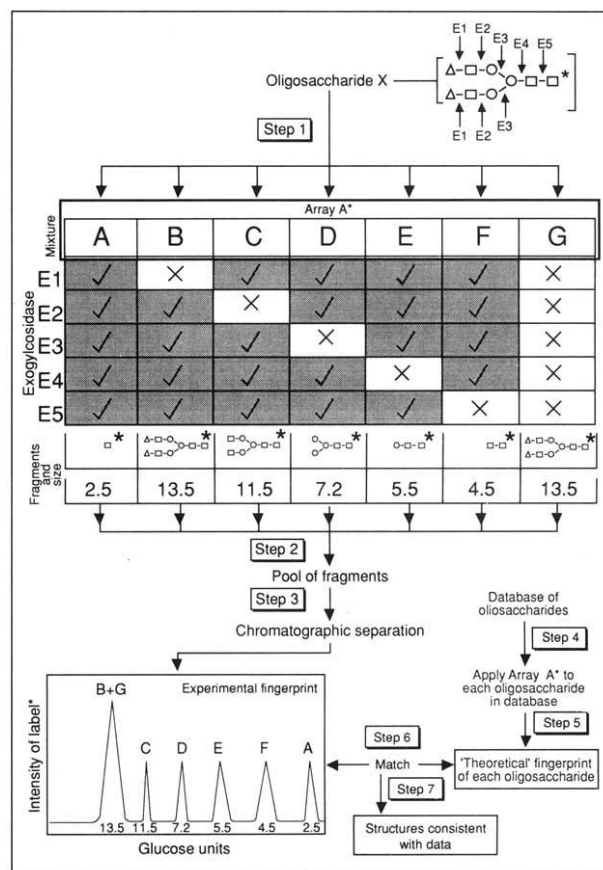
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Schematic representation of the reagent array analysis method. For clarity, a symbolic representation of an oligosaccharide (X) is given together with the points of cleavage within this oligosaccharide by enzymes E₁, E₂, E₃, E₄ and E₅. The symbols within structure X are as follows: □ = N-acetylglucosamine, □* = N-acetylglucosamine (tritium-labelled), O = mannose and Δ = galactose. The oligosaccharide X is divided into seven equal aliquots. Each aliquot is incubated with one of the enzyme mixtures A→G. The set of mixtures A→G constitutes the reagent array A*. After incubation, a fragment of oligosaccharide X is generated by each mixture, all fragments are combined, and a single chromatographic separation is performed. The experimental fingerprint is characterized by the relative intensity and hydrodynamic volume (measured in glucose units) of each fragment. In the example, letters within the experimental fingerprint indicate the enzyme mixtures from which that fragment was derived. The experimental fingerprint is matched to fingerprints generated by applying array A* to each oligosaccharide in the database. Steps 1–3 are performed experimentally and steps 4–7 through software. For practical examples of this method, see reference 3.



experimental data can be matched with a high degree of statistical confidence to the theoretical (and therefore effectively experimentally 'perfect') database. Conditions under which most of the currently used exoglycosidases retain full and independent activity have also been defined³. The method also has the advantage of providing internal checks on the activity of the enzymes used. This and other practical aspects are discussed more fully in ref. 3.

Future developments

It should be emphasized that RAAM has so far been shown to be applicable to the structural analysis of N-linked type oligosaccharides. In principle, however, it may be generalized to other 'families' of oligosaccharides, in which case different arrays would need to be developed. In addition to the advantages in terms of speed of analysis, a key feature of RAAM is its ease of automation. Products are anticipated to be available shortly that will consist of pre-set enzyme arrays, a chromatographic system and a full software package. These will allow a user to analyse initially N-linked type oligosaccharides using RAAM in a completely automated manner. In due course, extensions of the enzyme arrays and software package should allow other types of oligosaccharides to be similarly analysed. In

this way, the full value of enzymatic analysis of oligosaccharide structure should become broadly and easily accessible. These structural analysis products are expected to complement the existing array of physical and chemical methods already in place, and to add to the rapidly growing range of instrumentation that is available specifically for glycosylation analysis, notably the Dionex Bio LC (Sunnyvale, California, USA) and Oxford GlycoSystem's GlycoPrep 1000 and GlycoMap 1000 (Abingdon, Oxon, UK).

Christopher Edge, Thomas Rademacher, Mark Wormald and Raymond Dwek are members of the Glycobiology Institute, Oxford University, South Parks Road, Oxford OX1 3QU, UK, which is supported by Monsanto Company, and Raj Parekh is vice-president and director of research at Oxford GlycoSystems, Unit 4, Blacklands Way, Hitching Court, Abingdon Business Park, Abingdon, Oxon OX14 1RG, UK. For more information, fill in reader service number 100.

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What's hot and what's not in Washington

Next week's American Chemical Society meeting to be held in Washington, DC, USA will feature a collection of US patents on CD-ROM, a protein sequencer and software for the analysis of mass spectral data of proteins and peptides.

MILLIPORE has introduced a Uniplex **chemiluminescent DNA sequencing kit for thermal cycle sequencing** — a process that utilizes a thermostable DNA polymerase to amplify the DNA template during the sequencing procedure (*Reader Service No. 101*). With thermal cycle sequencing, only nanogram quantities of template are needed for sequencing, recalcitrant DNA structures can be sequenced through, and the 'pre-reaction' steps of denaturing double-stranded DNA and annealing primers are



Millipore's cycle sequencing kit.

eliminated. The kit contains the Vent (exo⁻) DNA polymerase from New England Biolabs, all the reaction mixes, and all the detection reagents, including biotinylated primers, biotinylated alkaline phosphatase, streptavidin and the chemiluminescent substrate. It can be used to sequence templates in a variety of standard sequencing vectors, such as M13, pUC, RD20, pBR322, lambda gt10, lambda gt11 and other commercially available vectors. Millipore says that the sensitivity of the chemiluminescent detection is equivalent to that of radioactivity, but the results are generated much more quickly — only 75 min after the completion of electrophoresis. See booth 1201.

MicroPatent introduces a complete Chemical PatentImages **collection of US chemical patents on CD-ROM**, which includes structures, formulas and diagrams (*Reader Service No. 102*). Chemical PatentImages eliminates the need for researchers to search through microfilm records or wait weeks while individual patents are delivered from the US Patent Office, the company says. A subscription for Chemical PatentImages provides users with biweekly discs, each containing over 1,000 of the latest chemical patents. To search for patents, Chemical PatentImages offers a range of options. Users can search on most bibliographic categories, including patent number, patent class, inventor or assignee. Boolean and phrase-searching capabilities allow the user to refine or expand the results of the search. A range of viewing tools is

available, including zoom, rotate, and page forward and back. Copies of patents can be printed using any laser printer. Current 1992 subscriptions are available for \$2,750 (US), with discs being updated on a biweekly basis. Backfiles to 1975 are available for \$3,000 to \$4,500 (US) per year, depending on how many years are ordered. Chemical PatentImages can be used on any PC-compatible computer equipped with a CD-ROM drive. See booth 534.

Coulter's 80-page **cytometry catalogue** features over 270 of the company's monoclonal antibodies, reagents, kits and instruments (*Reader Service No. 103*). Many new products are being introduced, including the three-colour marker, CD-3-ECD/T4-RD1/T8-FITC, with matching isotypic control. Additionally, the HIV p-24 antigen test kit is being released with the ICD-Prep, which dissociates complexed antigen, enabling laboratories to measure antigen previously undetectable (even in paediatric samples). For easy reference, a product number listing in numerical sequence has been included in the back of the catalogue. See booths 1124–1126

Protein chemistry

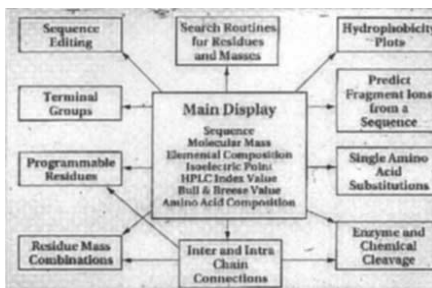
The HP G1005A **protein sequencing system** from Hewlett Packard is a complete, automated chemistry and analytical system for the sequence analysis of protein and peptide samples in picomole or nanomole amounts (*Reader Service No. 104*). The system consists of the protein sequencer, a sample-preparation station and an online phenylthiohydantion (PTH)-amino acid analyser based on the company's HP 1090M high-performance liquid chromatograph. The system controller, printer and consumables such as sequencing reagents, solvents and columns complete the system. The protein sequencer performs automated sequencing chemistry on a biphasic, packed-column sample support. The biphasic sequencer column allows direct loading of samples from complex matrices or from dilute solutions. This, Hewlett Packard says,



Protein sequencing system.

reduces the number of sample-handling steps and avoids sample loss. Sample solutions may include many contaminants, such as salts, amines, nonvolatile buffers, detergents and surfactants, which are incompatible with more traditional sequencing techniques. The HP G1005A's dual-sequencer columns allow back-to-back sequence runs to be performed without operator intervention. Reagents, solvents and gases all may be refilled while the sequencer is running, eliminating the need for 'downtime' between samples. The company says that the ternary HPLC system and chromatographic method can separate the 19 standard PTH-amino acids, sequencing byproducts and several modified forms of PTH-cysteine to greater than 98 per cent resolution. Initially the HP G1005A will be available in the United States; systems are expected to be available in Europe and selected regions in the rest of the world in 1993. See booth 1107.

Perkin-Elmer Sciex Instruments has introduced MacBioSpec, a new **software program that facilitates the analysis of mass spectral data of peptides and proteins** (*Reader Service No. 105*). MacBioSpec is incorporated into the PE Sciex API LC/MS operating software but is also offered as a standalone program. An enhanced version of MacProMass (a software program developed by the Beckman Research Institute of



MacBioSpec — software for the analysis of mass spectral data of proteins and peptides.

the City of Hope), MacBioSpec uses a graphical user interface and accommodates a variety of protein structures, including cyclic peptides and multiple chain proteins. The software program can calculate elemental composition, amino acid composition, isoelectric point, surface free energy and high-performance liquid chromatography values for whole structures and peptide fragments resulting from enzymatic or chemical degradations. It also provides a table of the common single- or multi-charged peptide fragment ions, as well as molecular mass calculations for positive and negative molecular ions. Perkin-Elmer says that

PRODUCT REVIEW

MassBioSpec facilitates the analysis of variant proteins with a subroutine that systematically catalogues single amino acid substitutions corresponding to mass differences between observed and expected molecular ions. Interchain and intrachain disulphide bonds and other types of linkages are maintained throughout chemical and enzymatic operations. The program runs on any Apple Macintosh computer with at least two megabytes of RAM and System 6.0 or higher. See booth 835.

Hyperbond from Beckman Instruments is a new **polymeric membrane** with properties that make it suitable for isolating samples for protein sequence analysis (*Reader Service No. 106*). Beckman says that, compared to polyvinylidene difluoride membranes, Hyperbond exhibits a much higher binding capacity during the blotting process and can even trap and retain very short peptides. In the sequencer, the properties of Hyperbond allow the Edman degradation to operate at high efficiency, without the need for special procedures.

■ Instrumentation update

The Jasco GR-980 **ternary gradient HPLC system** from Ciba Corning can be used for routine analyses as well as research applications (*Reader Service No. 107*). The system, which has a 15-cm wide footprint, comprises a ternary gradient pump, bottles and tray. For optimum flexibility, the system enables isocratic, binary and ternary mixing via three solvent channels, with a choice of flow or composition programming. Sixty-four steps are available for each run to accommodate the most complex of gradients, with the added facility of ten files for the storage of methods. Three, time-programmable event outputs allow column switching and control of external components. A timer fitted to the pump enables automatic switch-off after a predetermined

time and the stop/start mechanism allows control from an autosampler or data handling system. Jasco will be in booth 1036.

New literature is available on the **chromatography workstation package**, PL Caliber, from Polymer Laboratories, which has complementary GPC/SEC and LC/GC software modules (*Reader Service No. 108*). With PL Caliber, parameters controlling data collection, data handling and reporting of results are stored in 'methods' files. Once saved, a method can be quickly retrieved for future use, eliminating tedious repetition, the company says. A 'sample strip' facility provides a rapid method for storing and managing samples for analysis. Within a sample strip, sample-specific parameters can be quickly defined for each individual sample in the sequence. These parameters include the method to be used, the type of sample (calibrant or unknown) and other sample-related information. Once defined and stored, sample strips can be initiated to perform rapidly online analysis or re-analysis of individual or multiple samples. The software has been designed to collect data from up to six detectors, perform data analysis and generate reports simultaneously. Polymer Laboratories will be in booths 518-520.

Cell disruption



According to Microfluidics, its M-110Y Microfluidizer **cell disrupter** can double cell disruption recovery rates without damaging cell contents (*Reader Service No. 109*). The high-performance mixer can function at a maximum liquid pressure of 23,000 p.s.i. Difficult applications such as the disruption of yeast, fungi and spores can be processed under forces powerful enough to break cell walls, yet gentle enough to optimize output, Microfluidics says. The patented interaction chamber uses no moving parts and instead uses the forces of shear, cavitation and impact. The device can process sample sizes from 50 ml.

LDC Analytical's mercuryMonitor 3200 **detector** provides ultra-low detection limits to one part-per-thousand and a usable sensitivity range down to 0.0005 AU (*Reader Service No. 110*). The detector is an integral part of the company's complete mercury analysis system or, alternatively, can be purchased as a separate item for use with existing equipment. It utilizes a 10-cm pathlength cell and has, LDC says, excellent stability and dynamic linear range due to the dual-beam optical system. See booth 1127. □

These notes are compiled by Diane Gershon from information supplied by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

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